

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/00, 1/68, C12P 21/06, C12N 1/16, 1/20, 5/00, 9/02, 9/08, 15/00, C07H 17/00, 19/00, 21/00, A61K 38/00, 49/00	A1	(11) International Publication Number: WO 95/30766 (43) International Publication Date: 16 November 1995 (16.11.95)
--	-----------	---

(21) International Application Number: **PCT/US95/05744**
(22) International Filing Date: **8 May 1995 (08.05.95)**

(30) Priority Data:
08/238,821 6 May 1994 (06.05.94) **US**

(71) Applicant: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; NIH/OTT, Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US).

(72) Inventors: GOLDSTEIN, Joyce, A.; 10501 New Arden Way, Raleigh, NC 27613 (US). ROMKES-SPARKS, Marjorie; 4 Lockwood Road, Export, PA 15632 (US). DE MORAIS, Sonia, M., F.; Apartment M5, 6123 Farrington Road, Chapel Hill, NC 27514 (US).

(74) Agents: DOW, Karen, B. et al.; Townsend and Townsend Khourie and Crew, 20th floor, One Market Plaza, Steuart Street Tower, San Francisco, CA 94105 (US).

(81) Designated States: AU, CA, CN, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: **CLONING, EXPRESSION AND DIAGNOSIS OF HUMAN CYTOCHROME P450 2C19: THE PRINCIPAL DETERMINANT OF S-MEPHENYTOIN METABOLISM**

(57) Abstract

The invention provides two novel members of the cytochrome P450 2C subfamily of enzymes, designated 2C18 and 2C19. DNA segments encoding these enzymes are also provided. The 2C19 polypeptide represents the principal human determinant of human S-mephenytoin 4'-hydroxylase activity. The invention also provides methods of identifying drugs metabolized by S-mephenytoin 4'-hydroxylase activity. Drugs shown to be metabolized by this activity should in general not be administered to individuals having, or belong to an ethnic group at risk of, a polymorphic deficiency in S-mephenytoin 4'-hydroxylase activity. The invention also provides methods of diagnosing individuals having a polymorphic deficiency.

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LV	Latvia	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	MG	Madagascar	TJ	Tajikistan
DE	Germany	ML	Mali	TT	Trinidad and Tobago
DK	Denmark	MN	Mongolia	UA	Ukraine
ES	Spain			US	United States of America
FI	Finland			UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

5
CLONING, EXPRESSION AND DIAGNOSIS OF
HUMAN CYTOCHROME P450 2C19:
10 THE PRINCIPAL DETERMINANT OF S-MEPHENYTOIN METABOLISM

15
TECHNICAL FIELD

The present invention relates generally to isolation and exploitation of a novel member of the cytochrome P450 2C subfamily of enzymes 2C19, which is shown to be the principal human determinant of human S-mephenytoin metabolism. The invention also relates to the isolation and exploitation of an additional member of this family designated 2C18.

25 BACKGROUND OF THE INVENTION

The cytochromes P450 are a large family of hemoprotein enzymes capable of metabolizing xenobiotics such as drugs, carcinogens and environmental pollutants as well as endobiotics such as steroids, fatty acids and prostaglandins. Some members of the cytochrome P450 family are inducible in both animals and cultured cells, while other forms are constitutive. This group of enzymes has both harmful and beneficial activities. Metabolic conversion of xenobiotics to toxic, mutagenic and carcinogenic forms is a harmful activity. Detoxification of some drugs and other xenobiotic substances is a beneficial activity (Gelboin, *Physiol. Rev.* 60:1107-1). A further beneficial activity is the metabolic processing of some drugs to activated forms that have pharmacological activity.

40 Genetic polymorphisms of P450 enzymes result in phenotypically-distinct subpopulations that differ in their

ability to perform particular drug biotransformation reactions. These phenotypic distinctions have important implications for selection of drugs. For example, a drug that is safe when administered to most human may cause intolerable side-effects in an individual suffering from a defect in a P450 enzyme required for detoxification of the drug. Alternatively, a drug that is effective in most humans may be ineffective in a particular subpopulation because of lack of a P450 enzyme required for conversion of the drug to a metabolically active form. Accordingly, it is important for both drug development and clinical use to screen drugs to determine which P450 enzymes are required for activation and/or detoxification of the drug. It is also important to identify individuals who are deficient in a particular P450 enzyme.

A cytochrome P450 polymorphism of particular concern results in reduced levels of S-mephenytoin 4'-hydroxylase activity in certain subpopulations. (Küpfer et al., *Eur. J. Clin. Pharmacol.* 26:753-759 (1984); Wedlund et al., *Clin. Pharmacol. Ther.* 36:773-780 (1984). Two phenotypes, extensive and poor metabolizers, are present in the human population. Poor metabolizers are detected at low frequencies in Caucasians (2-5%) but at higher frequencies in the Oriental population (~20%) (Nakamura et al., *Clin. Pharmacol. Ther.* 38:402-408 (1985); Jurima et al., *Br. J. Clin. Pharmacol.* 19:483-487 (1985) and blacks (~12%). 4'-hydroxylation of S-mephenytoin is 3-10 fold higher than that of the R- enantiomer in extensive metabolizers, but the ratio is approximately 1 or less in poor metabolizers (Yasumori et al., *Mol. Pharmacol.* 35:443-449 (1990). Rates of S-mephenytoin 4'-hydroxylation in liver microsomes are also much higher than those of R-mephenytoin in extensive metabolizers.

There is some evidence that S-mephenytoin 4' hydroxylase activity resides in the cytochrome P450 2C family of enzymes. A number of 2C human variants (designated 2C8, 2C9 and 2C10) have been partially purified, and/or cloned. See Shimada et al., *J. Biol. Chem.* 261:909-921 (1986); Kawano et al., *J. Biochem. (Tokyo)* 102:493-501 (1987); Gut et al.,

Biochem. Biophys. Acta 884:435-447 (1986); Beaune et al.,
Biochem Biophys. Acta 840:364-370 (1985); Ged et al.,
Biochemistry 27:6929-6940 (1988)); Umbenhauer et al.,
Biochemistry 26, 1094-1099 (1987); Kimura et al., Nucleic
5 Acids Res. 15:10053-10054 (1987); Shephard et al., Ann. Humn.
Gentc. 53:23-31 (1989); Yasumori et al., J. Biochem. 102:1075-
1082 (1987); Relling et al., J. Pharmacol. Ther. 252:442-447.
A comparison of the P450 2C cDNAs and their predicted amino
acid sequences shows that about 70% of the amino acids are
10 absolutely conserved among the human P450 2C subfamily. Some
regions of human P450 2C protein sequences have particularly
highly conservation, and these regions may participate in
common P450 functions. Other regions show greater sequence
divergence regions and are likely responsible for different
15 substrate specificities between 2C members.

There has been considerable controversy as to
whether any of the known 2C members encodes the principal
human determinant of S-mephenytoin 4' hydroxylase activity, in
which the polymorphism discussed above presumably resides.
20 The multiplicity and common properties of cytochromes P450
make it difficult to separate their different forms,
especially the minor forms. Even in situations where P450
cytochromes have been isolated in purified form by
conventional enzyme purification procedures, they have been
25 removed from the natural biological membrane association and
therefore require the addition of NADPH-cytochrome P450
reductase and other cell fractions for enzymatic activity.

The known members of the cytochrome P450 2C family
exhibit only low-levels of S-mephenytoin 4'-hydroxylase
30 activity, if any. Moreover, such low levels of activity are
not specific for the S-enantiomer. For example, when the cDNA
isolated by Kimura et al. (1987), *supra*, was expressed in
HepG2 cells, it metabolized racemic and (R)-mephenytoin but
had no (S)-mephenytoin hydroxylase activity, suggesting that
35 the polymorphism in the metabolism of (S)-mephenytoin resides
in a different member of the P450 family. As a further
example, Yasumori et al. (1991), *supra*, reported that an
allelic variant of 2C9 (Arg¹⁴⁴Tyr³⁵⁸Iso³⁵⁹Gly⁴¹⁷) showed a low-

level of catalytic activity toward S-mephenytoin in a cDNA-directed yeast expression. However, Srivastava et al., *Mol. Pharmacol.* 40:69-69 (1991) expressed an identical cDNA in yeast and a Arg¹⁴⁴Cys³⁵⁸Iso³⁵⁹Asp⁴¹⁷ variant (2C10 by present nomenclature) but were unable to demonstrate catalytic activity of 2C9 or 2C10 toward S-mephenytoin. Relling et al., *J. Pharmacol. Exper. Ther.* 252:442-447 (1990), were also unable to demonstrate catalytic activity of an allelic variant of Cys¹⁴⁴Tyr³⁵⁸Ile³⁵⁹Gly⁴¹⁷-2C9 toward S-mephenytoin using a retroviral cDNA expression system in HepG2 cells. In contrast, all of these 2C9 variants metabolized tolbutamide in the various expression systems confirming that failure to observe S-mephenytoin 4'-hydroxylase activity was not due to deficiencies in the expression system.

Based on the foregoing, it is apparent that a need exists to identify and isolate the P450 2C family member representing the principal determinant of S-mephenytoin 4'-hydroxylase activity in humans. There is also a need for stable cell lines expressing the S-mephenytoin 4'-hydroxylase activity. A need is also apparent for methods of screening drugs for safety and efficacy in individuals deficient in S-mephenytoin 4'-hydroxylase activity. There is also a need for methods for diagnosing individuals deficient in S-mephenytoin 4'-hydroxylase activity. The present invention fulfills these and other needs.

SUMMARY OF THE INVENTION

The invention provides purified cytochrome P450 2C19 polypeptides. The amino acid sequence of an exemplary P450 2C19 polypeptide is designated SEQ. ID. No. 1. Other cytochrome P450 2C19 polypeptides usually comprises an amino acid sequence having at least 97% sequence identity with the exemplified sequence. Many of the 2C19 polypeptides of the invention exhibit stereospecific S-mephenytoin 4'-hydroxylase activity. The activity is typically at least about 1 nmol mephenytoin per nmol of the purified polypeptide per minute.

The invention also provides purified cytochrome P450 2C18 polypeptides. The amino acid sequences of exemplary 2C18 polypeptides are designated SEQ. ID. Nos. 5 and 11.

In another aspect of the invention, purified DNA segments encoding the P450 2C19 polypeptides described above are provided. Some DNA segments encode the exemplary P450 2C19 having the amino acid sequence designated SEQ. ID. No. 1. One such exemplary DNA segment is designated SEQ. ID. No. 2. Other DNA segments encode the P450 2C18 polypeptides described above. Exemplary DNA segments are designated SEQ. ID. Nos. 6 and 12.

In a further aspect of the invention stable cell lines are provided. The cell lines comprise an exogenous DNA segment encoding a cytochrome P450 2C19 polypeptide having at least 97% sequence identity with the amino acid sequence designated SEQ. ID. No. 1. The DNA segment is capable of being expressed in the cell line. Cell lines preferably produce high levels of the P450 2C19 polypeptide such as 10-200 pmol of the polypeptide per mg of total microsomal protein. Preferred cell lines are eukaryotic, including yeast and insect cells.

The invention also provides methods of producing a cytochrome P450 2C19 polypeptide. In these methods, a stable cell line, as described above, is cultured under conditions such that the DNA segment contained in the cell line is expressed.

The invention also provides antibodies that specifically bind to a 2C19 polypeptide comprising the amino acid sequence designated SEQ. ID. No. 1. Preferred antibodies are incapable of binding to nonallelic forms of 2C polypeptides, such as 2C9.

In another aspect, the invention provides methods of screening for a drug that is metabolized by S-mephenytoin 4'-hydroxylase activity. The drug is contacted with a cytochrome P450 2C19 polypeptide. A metabolic product resulting from an interaction between the polypeptide is detected. The presence of the product indicates that the drug is metabolized by the S-mephenytoin 4'-hydroxylase activity. The cytochrome P450

2C19 used in the methods may be substantially pure or may be a component of a lysate of a stable cell line. The cytochrome P450 2C19 polypeptide may also be a component of an intact stable cell line. Some methods further comprise the steps of contacting the drug with a liver extract comprising a mixture of cytochrome P450 polypeptides, and detecting a metabolic product resulting from an interaction between the drug and the mixture of cytochrome P450 polypeptides.

The invention also provides methods of identifying a mutagenic, carcinogenic or cytotoxic compound. In some methods, the compound is contacted with a stable cell line capable of expressing a 2C19 polypeptide, such as described above. Mutagenic, carcinogenic or cytotoxic effects of the compound on the cell line are assayed. In other methods, the compound is contacted with a cytochrome P450 2C19 polypeptide in a reaction mixture. A metabolic product is generated resulting from S-mephenytoin 4'-hydroxylase activity on the compound. The metabolic product is assayed for mutagenic, carcinogenic or cytotoxic effects on a test cell line. The effects indicate that the compound is mutagenic, carcinogenic or cytotoxic. In some methods, the test cell line is added to the reaction mixture before, during or after the contacting step. The 2C19 polypeptide used in these methods can be substantially pure or a component of a lysate of a stable cell line. The 2C19 polypeptide can also be a component of an intact stable cell line. *Salmonella typhimurium* is a preferred cell line.

The invention also provides methods for testing the chemopreventive activity of an agent. A stable cell line capable of expressing a 2C19 polypeptide, such as described above, is contacted with an agent suspected of being chemopreventive in the presence of a carcinogen. The agent can be contacted with the cell line before addition of the carcinogen. Effects of the agent on the cell line that are indicative of chemopreventive activity are monitored.

The invention also provides methods for determining the metabolites activated by a carcinogenic or xenobiotic. A stable cell line capable of expressing a 2C19 polypeptide,

such as described above, is contacted with the suspected carcinogen or xenobiotic. Metabolites and/or their effects are identified.

The invention also provides methods of detecting a cytochrome 2C19 polypeptide in a tissue sample. The tissue sample is contacted with an antibody that specifically binds to the 2C19 polypeptide preferably without specifically binding to nonallelic variants such as 2C9. Specific binding between the antibody and the polypeptide is detected to indicate the presence of the polypeptide.

In another aspect of the invention, methods of diagnosing a patient having a deficiency in S-mephenytoin 4'-hydroxylase activity are provided. In these methods, a sample of nucleic acids is obtained from the patient, and a cytochrome P450 2C19 DNA sequence from the nucleic acids in the sample is analyzed for the presence of a polymorphism indicative of the deficiency. The most frequently occurring polymorphisms in the P450 2C19 genes occur at nucleotides 681 and 636 of the 2C19 gene.

In some methods, the P450 2C19 DNA sequence subject to analysis is genomic. In such methods, an amplifying step is often primed from a forward primer sufficiently complementary with a first subsequence of the antisense strand of the 2C19 sequence to hybridize therewith, and a reverse primer sufficiently complementary to a second subsequence of the sense strand of the 2C19 sequence to hybridize therewith.

Some methods detect a polymorphism at nucleotide 681 of the coding region of the P450 2C19 DNA genomic sequence. This can be achieved by selecting a forward primer that hybridizes upstream from nucleotide 681 of the coding region, and a reverse primer that hybridizes downstream from nucleotide 681 of the coding region. Amplification products generated from these primers can be analyzed by digesting the amplified DNA segment with a restriction enzymes that recognizes a site that includes nucleotide 681 of the coding region.

Other methods detect a polymorphism at nucleotide 636 of the coding region of the P450 2C19 DNA genomic

sequence. This can be achieved using a forward primer that hybridizes upstream from nucleotide 636 of the coding region, and a reverse primer that hybridizes downstream of nucleotide 636 of the coding region. Amplification products are
5 conveniently analyzed by digestion with an enzyme that recognizes a site that includes nucleotide 636 of the coding region.

Other methods detect the 681 polymorphism by a different approach involving selective amplification of the
10 wildtype or mutant allele. For example, for selective amplification of the wildtype allele, a suitable forward primer has about 10-50 contiguous nucleotides from the wildtype 2C19 sequence shown in Fig. 16 including the nucleotide at position 681 of the coding region. The forward
15 primer primes amplification from the complement of the wildtype 2C19 sequence without priming amplification from the complement of the mutant 2C19 sequence shown in Fig. 16. Preferably, the 3' nucleotide of the forward primer is the nucleotide at position 681. Analogously, the 681 mutant
20 allele can be amplified using a forward primer having about 10-50 contiguous nucleotides from the mutant 2C19 sequence shown in Fig. 16 including the nucleotide at position 681 of the coding sequence. The forward primer primes amplification from the complement of the mutant 2C19 sequence
25 without priming amplification from the complement of the wildtype 2C19 sequence shown in Fig 16.

The invention also provides analogous methods for detection of the 636 polymorphism.

In other methods, the segment of 2C19 DNA subject to
30 analysis is a cDNA sequence. cDNA is produced by reverse transcribing mRNA in the sample to produce the cDNA sequence. In some methods for detecting the 681 polymorphism, the forward primer comprises about 10-50 contiguous nucleotides upstream of nucleotide 643 of the coding region of the
35 wildtype 2C19 cDNA sequence shown in Fig. 12 and hybridizes to the complement of the 2C19 sequence upstream from nucleotide 643 of the coding region, and the reverse primer comprises about 10-50 contiguous nucleotides from the complement of the

wildtype 2C19 cDNA sequence shown in Fig. 12 and hybridizes to the 2C19 sequence downstream from nucleotide 682 of the coding region. In other methods, the forward primer hybridizes to the complement of the wildtype 2C19 cDNA sequence shown in Fig. 12 between nucleotides 643 and 682 without hybridizing to the complement of the mutant 2C19 cDNA sequence shown in Fig. 12. In other methods, the reverse primer hybridizes to the wildtype 2C19 cDNA sequence shown in Fig. 12 between nucleotides 643 and 682 without hybridizing to the mutant 2C19 cDNA sequence shown in Fig. 12.

The invention provides analogous methods for diagnosing the 636 polymorphism from cDNA. In some methods, the forward primer comprises about 10-50 contiguous nucleotides upstream of nucleotide 636 of the coding region of the wildtype 2C19 cDNA sequence shown in Fig. 12, and the reverse primer comprises about 10-50 contiguous nucleotides from the complement of the wildtype 2C19 cDNA sequence shown in Fig. 12 downstream from nucleotide 636 of the coding region.

The invention also provides methods capable of detecting any polymorphism from cDNA. In these methods, the full-length 2C19 cDNA sequence is usually amplified. Analysis is often performed by sequencing a segment of the 2C19 cDNA amplification product.

The invention provides further methods for diagnosing polymorphisms in genomic DNA. In these methods, genomic DNA is digested with a restriction enzyme that recognizes a site that includes nucleotide 636 or 681 of the coding region. The digestion products are then detected by Southern blotting with a labelled segment of the 2C19 DNA sequence as a probe.

In another aspect of the invention, diagnostic kits are provided. Some diagnostic kits comprise forward and reverse primers. The forward primer is sufficiently complementary with a first subsequence of the antisense strand of a double-stranded 2C19 genomic DNA sequence to hybridize therewith, and the reverse primer sufficiently complementary with a second subsequence of the sense strand of the 2C19

genomic sequence to hybridize therewith. For example, in some methods for diagnosis of the 681 polymorphism, the first subsequence is upstream of nucleotide 681 of the coding region, and second subsequence is downstream of nucleotide 681 of the coding region. Similarly, in some methods for diagnosis of the 636 polymorphism, the first subsequence is upstream of nucleotide 636 of the coding region, and the second subsequence is downstream of nucleotide 636 of the coding region.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows Western blots of human liver microsomal proteins. Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis. Blot A was performed using polyclonal antibody to 2C9 and blot B with anti-2C8 (HLx). Each lane represents 20 μ g of microsomal protein from an individual liver. The 2C8 antibody also recognized purified rat P450 2C13(g). cDNA libraries were constructed from livers 860624 (low HLx) and S33 (high HLx).

Figure 2 contains nucleotide sequences of human P450 2C cDNAs. 2c (SEQ. ID. No. 14) is indicated in the top line and represents the consensus sequence where information from more than one sequence is available. Sequences were determined by the dideoxy chain termination method. The differences observed for clones 25 (SEQ. ID. No. 4) and 65 (SEQ. ID. No. 10) are underlined. The termination codons are starred. The heme binding region and polyadenylation signals are underlined. The one-base difference between 29c (SEQ. ID. No. 6) and 6b (SEQ. ID. No. 12) are also underlined. The termination codon is starred. The new allelic variant proteins of 2C18, referred to as 29c (SEQ. ID. No. 5) and 6b (SEQ. ID. No. 11), and the new protein of 2C19, referred to as 11a (SEQ. ID. No. 1), are compared with the protein of 2C8, referred to as 2C8 (SEQ. ID. No. 7), and the allelic variant proteins of 2C9, referred to as 65 (SEQ. ID. No. 9) and 25 (SEQ. ID. No. 3).

Figure 3 depicts a comparison of amino acid sequences of cytochrome P450 2C8 allelic variants.

Figure 4 depicts a Western blot of recombinant transformed COS-1 cells. Each lane represents microsomal protein (50 μ g) from an independent transformation with the indicated P450 2C cDNA, mock-transfected cells (CON), 20 μ g of human liver microsomal protein (liver S5), or 2 pmol of pure P450g (2C13).

Figure 5 shows a Northern blot of human mRNAs. Each lane represents 10 μ g of mRNA, and the blot was probed with end-labeled T300R, an oligoprobe specified for 2C8 (SEQ. ID. No. 8) (top), stripped, and reprobed with 32 P-actin cDNA (bottom).

Figure 6: Western blots of yeast microsomes expressing recombinant P450 2C cDNAs. CON=control (yeast microsomes lacking recombinant proteins).

Figure 7: Linearity of S-mephenytoin 4'-hydroxylase activity and amount of recombinant cytochrome P450 2C19.

Figure 8: S-mephenytoin 4'-hydroxylase activity as a function of the molar ratio of cytochrome b_5 to recombinant cytochrome P450.

Figure 9: HPLC radiochromatograms of metabolites formed after incubation of labelled mephenytoin with P450 2C enzymes, human liver microsomes and yeast control.

Figure 10: Comparison of liver content of cytochrome P450 2C enzymes with S-mephenytoin 4'-hydroxylase activity. The upper part of the figure shows Western blots of liver samples from 16 individuals. The lower part of the figure shows the S-mephenytoin 4'-hydroxylation activity and ratios of S/R mephenytoin 4'-hydroxylase activity in each sample.

Figure 11: Correlation between hepatic 2C19 content and S-mephenytoin hydroxylase activity based on the data shown in Figure 10.

Figure 12: Sequence alignment of PCR products from normal and aberrantly spliced CYP2C19 cDNAs (SEQ. ID. Nos. 45 and 47), with the corresponding amino acid translations (SEQ. ID. Nos. 46 and 48) indicated above and below the nucleotide sequence. The new termination codon TAA in the aberrant cDNA is indicated by the word END and the asterisk. The PCR

primers are indicated by the horizontal arrows in the sequence. The aberrant CYP2C19 cDNA is missing 40 base pairs of the cDNA in poor metabolizers as indicated by the dotted line.

5 Figure 13: A. Diagram of strategy to amplify CYP2C19 cDNA transcripts from human liver samples. The sequence for the PCR primers is indicated in Fig. 12. This strategy yielded a 284 bp band for the normal cDNA, a 244 bp band for the aberrant cDNA and both bands with cDNA from
10 heterozygous individuals. The hatched area indicates the 40 bp deleted in exon 5 of the aberrant cDNA. B. Relation between genotype as assessed by reverse transcription PCR (RT-PCR) of human liver mRNA, CYP2C19 protein estimated by immunoblotting, S-mephenytoin hydroxylation activity, and the
15 ratio of metabolism of the R/S enantiomers. In vitro phenotype was based on high (E), intermediate (I) or low (P) S-mephenytoin 4'-hydroxylase activity.

 Figure 14: A. Diagram showing strategy used to genotype genomic DNA from human blood. B. Diagram of family
20 of propositus 61 (arrow) showing the pedigree and the gel of SmaI-digested PCR products. C. Analysis of genomic DNA from selected Caucasians subjects from United States or from Switzerland. The phenotype (EM, IM or PM) is indicated in the brackets above the gel. D. Analysis of genomic DNA from
25 selected Oriental subjects.

 Figure 15: A. Partial sequence of the intron 4/exon 5 junction of CYP2C19 in extensive and poor
metabolizers (SEQ. ID. Nos. 49 and 50). Intron sequences are shown in lower case and exon sequences in capitals. The
30 nucleotides deleted in the aberrantly spliced cDNA are indicated in bold. The polymorphic SmaI site is underlined in 2C19(wt). The highly conserved AG residues at the intron/exon junction are shown in black boxes. The consensus sequence (11YNCAGG) (Y=pyrimidine, R=purine, N=any base) for the 3'-
35 splice site is indicated underneath the normal and cryptic splice junctions. The branch point consensus sequence (CURAY) is placed underneath two putative branch points. B. Sequencing of PCR products of genomic DNA from three

individuals who were homozygous normal, heterozygous, and homozygous defective (based on their SmaI restriction digests). The polymorphic SmaI restriction site is indicated by the bracket in the homozygous wt sequence. The G→A base pair change corresponding to position 681 of the cDNA is also indicated. C. Schematic representation of splicing in CYP2C19_{wt} and in CYP2C19_m. The black box indicates the 40 bp that are deleted in exon 5 of poor metabolizers.

Figure 16: Additional 2C19 genomic sequence flanking the 681 polymorphism. The wildtype (SEQ. ID. No. 51) and mutant (SEQ. ID. No. 61) sequences are identical except for the G/A transposition at nucleotide 681. Regions of sequence ambiguity are indicated in lower case (n=any nucleotide, k=G/T ambiguity, r=A/G ambiguity, m=A/C ambiguity).

Figure 17: Genomic DNA sequence flanking the 636 polymorphism (also referred to as m2). Wildtype and mutant sequences are designated SEQ. ID. Nos. 52 and 54 respectively. Intron sequences are indicated in lower case and exons in capital. Translated amino acids (SEQ. ID. No. 53) are indicated above the nucleotide sequence. The numbers underneath the sequences indicate the first (482) and last (642) nucleotides in exon 4. The two mutations found in exon 4 are indicated in bold. The aberrant stop codon is indicated by the word "End." Exemplary primers for PCR amplification are underlined.

Figure 18: Diagnosis of 636 mutation in 2C19. The position of the PCR primers is indicated by arrows at 79-55 base pairs in intron 3 and 70-89 bp in intron 4. The size of the PCR products expected in the wild type gene (wt) and the size of the product in the 636 mutant allele are shown in the bottom lines.

Figure 19: Simultaneous detection of the 636 and 681 mutations.

DEFINITIONS

Abbreviations for the twenty naturally occurring amino acids follow conventional usage (*Immunology - A Synthesis* (E.S. Golub & D.R. Gren, eds., Sinauer Associates,

Sunderland, MA, 2nd ed., 1991) (hereby incorporated by reference for all purposes). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α -disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ -carboxyglutamate, ϵ -N,N,N-trimethyllysine, ϵ -N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω -N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). In the polypeptide notation used herein, the left-hand direction is the amino terminal direction and the right-hand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the lefthand end of single-stranded polynucleotide sequences is the 5' end; the lefthand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand that are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand that are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences".

The phrase "polynucleotide sequence" refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. It includes self-replicating plasmids, infectious polymers of DNA or RNA and non-functional DNA or RNA.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence

listing, such as a polynucleotide sequence shown in SEQ. ID. NO.2 or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (*i.e.*, a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith & Waterman, *Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Natl. Acad. Sci. (USA)* 85:2444 (1988), by computerized implementations of these algorithms (FASTDB (Intelligenetics), BLAST (National Center for Biomedical Information) or GAP, BESTFIT, FASTA, and TFASTA (Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI)), or by inspection, and the best alignment (*i.e.*, resulting in the highest percentage of sequence similarity over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (*i.e.*, on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of

sequence identity" (also sometimes referred to as "percentage homology") is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 96 percent sequence identity, more usually at least 97, 98 or 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length sequence of SEQ. ID. Nos. 2, 6 or 12.

As applied to polypeptides, the term "substantial identity" (or "substantial homology") means that two peptide sequences, when optimally aligned, such as by the programs BLAZE (Intelligenetics) GAP or BESTFIT using default gap weights, share at least 85% sequence identity preferably at least 96 percent sequence identity, more preferably at least 97, 98 or 99 percent sequence identity or more (e.g., 99.5 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is

serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "epitope" includes any protein determinant capable of specific binding to an immunoglobulin or T-cell receptor. Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

Specific binding exists when the dissociation constant for a dimeric complex is $\leq 1 \mu\text{M}$, preferably $\leq 100 \text{ nM}$ and most preferably $\leq 1 \text{ nM}$.

5 The term "allelic variants" refers to gene sequences mapping to the same chromosomal location in different individual in a species but showing a small degree of sequence divergence from each other. Typically, allelic variants encode polypeptides exhibiting at least 96% or 97% amino acid sequence identity with each other.

10 The term "nonallelic variants" refers to gene sequences that show similar structural and/or functional properties but map at different chromosomal locations in an individual. In the 2C family, nonallelic variants typically exhibit 70-96% amino acid sequence identity with each other.

15 The term "cognate variants" refers to gene sequences that are evolutionarily and functionally related between humans and other species such as primates, porcines, bovines and rodents such as mice and rats. Thus, the cognate primate gene to a human 2C19 gene is the primate gene which encodes an expressed protein which has the greatest degree of sequence identity to the 2C19 protein and which exhibits an expression pattern similar to that of the 2C19 protein.

20 Stringent conditions are sequence dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe.

25 Typically, stringent conditions will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60°C . As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one.

30

35

A polymorphism is a condition in which two or more different nucleotide sequences coexist in the same interbreeding population in a DNA sequence.

The term "oligonucleotide" refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, such as primers, probes, nucleic acid fragments to be detected, and nucleic acid controls. The exact size of an oligonucleotide depends on many factors and the ultimate function or use of the oligonucleotide. Oligonucleotides can be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences and direct chemical synthesis by a method such as the phosphotriester method of Narang et al., *Meth. Enzymol.* 68:90-99 (1979); the phosphodiester method of Brown et al., *Meth. Enzymol.* 68:109-151 (1979); the diethylphosphoramidite method of Beaucage et al., *Tetrahedron Lett.* 22:1859-1862 (1981); and the solid support method of U.S. Patent No. 4,458,066.

A primer is an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, i.e., in the presence of four different nucleoside triphosphates and an agent for polymerization (i.e., DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature.

"Probe" refers to an oligonucleotide which binds through complementary base pairing to a subsequence of a target nucleic acid. Probes will typically hybridize to target sequences lacking complete complementarity with the probe sequence on reducing the stringency of the hybridization conditions. The probes are preferably directly labelled as with isotopes or indirectly labelled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the target.

"Subsequence" refers to a sequence of nucleic acids that comprise a part of a longer sequence of nucleic acids.

The term "target region" refers to a region of a nucleic acid to be analyzed such as a polymorphic region.

Hybridization refers to binding between an oligonucleotide and a target sequence via complementary base pairing to achieve the desired priming by PCR polymerases or detection of hybridization signal, and sometimes embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization conditions.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

The invention provides novel cytochrome P450 2C polypeptides, DNA fragments encoding these polypeptides and cell lines expressing the polypeptides. The invention also provides methods of using the novel polypeptides for, *inter alia*, identifying drugs metabolized by S-mephenytoin 4'-hydroxylase activity.

I. Polypeptides

In one embodiment, the invention provides novel cytochrome P450 2C polypeptides, designated 2C18 and 2C19. The 2C18 and 2C19 proteins are nonallelic with each other and with known 2C polypeptides. An exemplary 2C19 polypeptide has the amino acid sequence designated SEQ. ID. No. 1. The invention also provides allelic variants of the exemplified 2C19 polypeptide, and natural and induced mutants of such variants. The invention provides human 2C19 polypeptides and cognate variants thereof. Typically, 2C19 variants exhibit at substantial sequence identity (e.g., at least 96% or 97% amino acid sequence identity) with the exemplified 2C19 polypeptide and cross-react with antibodies specific to this polypeptide. 2C19 variants are usually encoded by nucleic acids that show substantial sequence identity (e.g., at least 96% or 97% sequence identity) with the nucleic acid encoding the exemplified 2C19 variant (SEQ. ID. No. 2).

Some 2C19 polypeptides, including the exemplified polypeptide, exhibit high levels of stereospecific S-mephenytoin 4'-hydroxylase activity. See Table IV. Indeed, it is highly probable that 2C19 represents the principal human

determinant of this activity. Typically such 2C19 polypeptides exhibit a stereospecific S-mephenytoin 4'-hydroxylase activity of about 0.5-100, 1-10 or about 4-6 nmol S-mephenytoin per nmol 2C19 polypeptide per minute.

5 Frequently, the activity of 2C19 polypeptides is higher than of native human liver microsomes. The activity of such polypeptides for the R-enantiomer of mephenytoin is typically at least 10, 50 or 100-fold lower.

Other 2C19 polypeptides may lack substantial
10 stereospecific S-mephenytoin 4'-hydroxylase activity. Such polypeptides represent allelic variants of the exemplified 2C19 polypeptide. These polypeptides sometimes exhibit low levels of mephenytoin 4'-hydroxylase activity (i.e., less than about 0.5 or 0.2 nmol mephenytoin per nmol 2C19 polypeptide
15 per minute). This activity may, or may not be, stereospecific. Although the presence of a 2C19 polypeptide with low enzymic activity could account for the phenotype of a few individuals defective in S-mephenytoin 4'-hydroxylase activity, the phenotype in most such individuals results from
20 a complete or substantial absence of 2C19 polypeptide. See, e.g., Figure 10.

The invention also provides 2C18 polypeptides. The amino acid sequences of two allelic variants of 2C18 are designated SEQ. ID. Nos. 5 and 11. Also provided are allelic
25 variants of the exemplified 2C18 polypeptides, conjugated variants thereof, and natural and induced mutants of any of these. Typically, 2C18 variants exhibit substantial sequence identity (e.g., at least 96% or 97% amino acid sequence identity) with the exemplified 2C18 polypeptides and cross-
30 react with antibodies specific to these polypeptides. 2C18 variants are usually encoded by nucleic acids that show substantial sequence identity (e.g., at least 96% or 97% sequence identity) with the nucleic acid encoding the exemplified 2C18 variants (SEQ. ID. Nos. 6 and 12).

35 2C18 polypeptides typically show low levels of mephenytoin 4'-hydroxylase activity (0.01-0.2 nmol mephenytoin per nmol 2C18 polypeptide per min. For some 2C18 polypeptides, the activity shows a small degree of

stereoselectivity (up to about five fold). However, by contrast to the 2C19 polypeptides, such stereoselectivity as is shown by 2C18 polypeptides is in favor of the R enantiomer. Some variants of 2C18 show high levels of a distinct enzymic activity, namely, tolbutamide hydroxylase activity (e.g.,
5 about 50-200 pmol tolbutamide per nmol 2C18 polypeptide per min). Conceivably, some variants of 2C18 exhibit novel enzymic or regulatory functions not shared by other 2C family members.

10 Besides substantially full-length polypeptides, the present invention provides fragments of full-length 2C18 and 2C19 polypeptides. Some such fragments share the enzymic activity of a full-length fragment. A segment of a full-length 2C18 or 2C19 polypeptide will ordinarily comprise at
15 least 50 contiguous amino acids and more usually, 100, 200 or 400 contiguous amino acids from one of the exemplified polypeptide sequences, designated SEQ. ID. Nos. 1, 5 and 11. Fragments of full-length 2C18 and 2C19 polypeptides are often terminated at one or both of their ends near (i.e., within
20 about 5, 10 or 20 aa of) the boundaries of functional or structural domains. Fragments are useful for, inter alia, generating antibodies specific to a 2C19 or 2C18 polypeptide. Fragments consisting essentially of the hypervariable regions of these polypeptides are preferred immunoglobulins for
25 generating antibodies specific to a particular allelic variant.

II. Nucleic Acid Fragments

In another aspect of the invention, nucleic acids
30 fragments are provided. An exemplified cDNA sequence of a 2C19 polypeptide is designated SEQ. ID. No. 2. Exemplified cDNA sequences encoding two variant 2C18 polypeptides are designated SEQ. ID. Nos. 6 and 12. The exemplified sequences include both translated regions and 3' and 5' flanking
35 regions. The exemplified sequence data can be used to design probes for other DNA fragments encoding 2C18 or 2C19 polypeptides (or fragments thereof). These DNA fragments include human genomic clones, cDNAs and genomic clones from

other species, allelic variants, and natural and induced mutants of any of these. Specifically, all nucleic acid fragments encoding all 2C18 and 2C19 polypeptides disclosed in this application are provided. Genomic libraries of many species are commercially available (e.g., Clontech, Palo Alto, CA), or can be isolated de novo by conventional procedures. cDNA libraries are best prepared from liver extracts.

The probes used for isolating clones typically comprise a sequence of about at least 15, 20 or 25 contiguous nucleotides (or their complement) of an exemplified DNA sequence (i.e., SEQ. ID. Nos. 2, 6 or 12). Preferably probes are selected from regions of the exemplified sequences that show a high degree of variation between different 2C nonallelic variants. Hypervariable regions are the nucleic acids encoding amino acids 181-210, 220-248, 283-296 and 461-479. Probes from these regions are likely to hybridize to allelic variants but not to nonallelic variants of the exemplified sequences under stringent conditions. Allelic variants can be isolated by hybridization screening of plaque lifts (Benton & Davis, *Science* 196:180 (1978)). Alternatively, cDNAs can be prepared from liver mRNA by polymerase chain reaction (PCR) methods. 5'- and 3'- specific primers for 2C19 are designed based on the nucleotide sequence designated SEQ. ID. No. 2. See generally *PCR Technology: Principles and Applications for DNA Amplification* (ed. H.A. Erlich, Freeman Press, NY, NY, 1992); *PCR Protocols: A Guide to Methods and Applications* (eds. Innis, et al., Academic Press, San Diego, CA, 1990); Mattila et al., *Nucleic Acids Res.* 19:4967 (1991); Eckert et al., *PCR Methods and Applications* 1:17 (1991); *PCR* (eds. McPherson et al., IRL Press, Oxford); and U.S. Patent 4,683,202 (each of which is incorporated by reference for all purposes).

Nucleotide substitutions, deletions, and additions can be incorporated into the polynucleotides of the invention. Nucleotide sequence variation may result from degeneracy of the genetic code, from sequence polymorphisms of 2C18 and 2C19 alleles, minor sequencing errors, or may be introduced by random mutagenesis of the encoding nucleic acids using

irradiation or exposure to EMS, or by changes engineered by site-specific mutagenesis or other techniques. See Sambrook et al., *Molecular Cloning: A Laboratory Manual* (C.S.H.P. Press, NY 2d ed., 1989) (incorporated by reference for all purposes).

III. Cell Lines

In another embodiment of the invention, cell lines capable of expressing the nucleic acid segments described above are provided. Stable cell lines are preferred to cell lines conferring transient expression. Stable cell lines can be passaged at least fifty times without reduction in the level of 2C polypeptides expressed by the cell lines. Preferably, cell lines are capable of being cultured so as to express 2C polypeptides at high levels, usually at least 0.2, 1, 10, 20, 50, 100, 200 or 500 pmol of 2C polypeptide per mg of microsomal protein. For example, the 2C19 expression level of many cell lines of the invention is typically about 0.2-10,000, 1-200, 7-100, 10-50 or 10-20 pmol 2C19 polypeptide per mg microsomal protein. An expression level of 10 pmol 2C19 per mg microsomal protein means that 2C19 represents about 0.06% of total cellular protein. For *E. coli* and insect cell lines, the recombinant P450 protein can comprise 5-10% of total cellular protein. Often, the stable cell lines of the invention express more than one P450 polypeptide. These cell lines express 2C18 and/or 2C19 together with other members of the 2C family, or other P450 cytochromes such as 1A1, 1A2, 2A6, 3A3, 3A4, 2B6, 2B7, 2C9, 2D6, and/or 2E1.

E. coli is one prokaryotic host useful for cloning the polynucleotides of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. Expression vectors typically contain expression control sequences compatible with the host cell, e.g., an origin of replication, any of a variety of well-known promoters, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda.

Vectors often also contain an operator sequence and/or a ribosome binding site. The control sequences are operably linked to a P450 DNA segment so as to ensure its expression and control the expression thereof.

5 Other microbes, such as fungi, particularly, yeast, are particularly useful for expression. *Saccharomyces* is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an
10 origin of replication, termination sequences and the like as desired. For example, the plasmid pAAH5 can be used. The 5'-noncoding sequence of the P450 2C cDNAs can be eliminated and six adenosines added by polymerase chain reaction (PCR) amplification to optimize expression in yeast cells. The 5'-
15 and 3'-primers recommended for amplification of 2C18 are 5'-GCAAGCTTAAAAAATGGATCCAGCTGTGGCTCT-3' (SEQ. ID. No. 15) and 5'-GCAAGCTTGCCAAACTATCTGCCCTTCT-3' (SEQ. ID. No. 16). This includes addition of a Hind III restriction site at both ends to allow insertion into the pAAH5 vector and six 6 adenosines
20 at the 5'-end to optimize translation. The final 20 bases of each sequence is specific for 20 bases at the 5'-end of 2C18 starting with the ATG for methionine and 20 bases of the 3'-noncoding region. The primers for 2C19 can be constructed similarly. The yeast strain used, *Saccharomyces cerevisiae*
25 334, can be propagated non-selectively in YPD medium (1% yeast extract, 2% peptone, 2% dextrose (Hovland et al. (1989) *Gene* 83, 57-64) and Leu+ transformants selected on synthetic minimal medium containing 0.67% nitrogen base (without amino acids), 0.5% ammonium sulfate, 2% dextrose and 20 µg/ml L
30 histidine (SD+His). Plates are made by the addition of 2% agar. Yeast can be transformed by the lithium acetate method of Ito et al. (1983) *J. Bacteriol.* 153, 163 and selected on SD+His for selection of transformants. Cells are then grown to mid-logarithmic phase (Oeda et al., *DNA* 4:203-210 (1985))
35 and microsomes containing recombinant protein can be prepared.

Insect cells (e.g., SF9) with appropriate vectors, usually derived from baculovirus, are also suitable for

expressing 2C polypeptides. See Luckow, et al. *Bio/Technology* 6:47-55 (1988) (incorporated by reference for all purposes).

Mammalian tissue cell culture can also be used to express and produce the polypeptides of the present invention (see Winnacker, *From Genes to Clones* (VCH Publishers, N.Y., N.Y., 1987). Suitable host cell lines include CHO cell lines (e.g., V79) (Dogram et al. (1990) *Mol. Pharmacol.* 37, 607-613), various COS cell lines, HeLa cells, myeloma cell lines and Jurkat cells, hepatoma cell lines (Hep G2), and a lymphoblastoid cell line AHH-1 TK+/- . Crespi et al. (1991) *Carcinogenesis* 12, 355-359. Expression vectors for these cells (e.g., pEBVHistK or pSV2) can include expression control sequences, such as an origin of replication, a promoter (e.g., a HSV tk promoter or *pgk* (phosphoglycerate kinase promoter), an enhancer (Queen et al., *Immunol. Rev.* 89:49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine papillomavirus, and the like. Expression control sequences are operably linked to a DNA segment encoding a P450 polypeptide so as to ensure the polypeptide is expressed.

The vectors containing the polynucleotide sequences of interest can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, calcium chloride transfection is commonly utilized for prokaryotic cells, whereas calcium phosphate treatment or electroporation may be used for other cellular hosts. (See generally Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Press, 2nd ed., 1989) (incorporated by reference in its entirety for all purposes).

Once expressed, the polypeptides of the invention and their fragments can, if desired, be purified according to standard procedures of the art, including ammonium sulfate precipitation, affinity columns, column chromatography, gel

electrophoresis and the like (see generally Scopes, *Protein Purification* (Springer-Verlag, N.Y., 1982).

IV. Antibodies

5 The invention also provides antibodies that specifically bind to epitopes on the 2C18 and 2C19 polypeptides of the invention. Some antibodies specifically bind to one member of the 2C family (e.g., 2C19) without binding to nonallelic forms. Some antibodies specifically
10 bind to a single allelic form of a 2C member such as the 2C19 polypeptide having the amino acid sequence designated SEQ. ID. No. 1. Antibodies that specifically bind to a 2C19 polypeptide without binding to a 2C9 polypeptide are particularly useful in view of the relatively high degree of
15 sequence identity between these nonallelic variants. See Table II. The production of non-human monoclonal antibodies, e.g., murine, lagomorpha, equine is well known and can be accomplished by, for example, immunizing an animal with a preparation containing a 2C19 polypeptide or an immunogenic
20 fragment thereof. Human antibodies can be prepared using phage-display technology. See, e.g., Dower et al., WO 91/17271 and McCafferty et al., WO 92/01047 (each of which is incorporated by reference in its entirety for all purposes). Humanized antibodies are prepared as described by Queen et
25 al., WO 90/07861.

V. Methods of Use

A. Identification of Drugs Unsuitable for Administration to Poor Metabolizers of S-Mephenytoin

30 The identification of a 2C19 polypeptide as the principal determinant of human S-mephenytoin 4'-hydroxylase activity facilitates methods of screening drugs that are metabolized by this enzyme. Such drugs likely lack efficacy and/or show intolerable side effects in individuals having a
35 defect in S-mephenytoin 4'-hydroxylase activity (low producers). The substantial absence of this activity in low producers often results in an inability to detoxify such drugs, preventing their elimination from the body.

Substantial absence of S-mephenytoin 4'-hydroxylase activity can also prevent metabolic processing of certain drugs to activated forms. Drugs suspected of being metabolized by S-mephenytoin 4'-hydroxylase activity include, in addition to
5 mephenytoin itself, omeprazole, proguanil, diazepam and certain barbiturates.

Drugs are screened for metabolic processing by S-mephenytoin 4'-hydroxylase activity in a variety of assays. See Example 5. In brief, the drug under test is usually
10 labelled with a radioisotope or otherwise. The drug is then contacted with a 2C19 polypeptide exhibiting S-mephenytoin 4'-hydroxylase activity (e.g., the polypeptide designated SEQ. ID. No. 1). The 2C19 polypeptide can be in purified form or can be a component of a lysate of one of the cell lines
15 discussed in Section III. Often, the 2C19 polypeptide is part of a microsomal fraction of a cell lysate. The 2C19 polypeptide can also be a component of an intact cell as many drugs are taken up by such cells. Often, the reaction mixture is supplemented with one or more of the following reagents:
20 dilauroylphosphatidylcholine, cytochrome P450 reductase, human cytochrome b5, and NADPH. (See Example 5, for concentrations of these reagents and a suitable buffer). After an incubation period (e.g., 30 min), the reaction is terminated, and centrifuged. The supernatant is analyzed for metabolic
25 activity, e.g., by a spectrographic or chromatographic method. The assay is usually performed in parallel on a control reaction mixture without a 2C19 polypeptide. Metabolic activity is shown by a comparative analysis of supernatants from the test and control reaction mixtures. For example, a
30 shift in retention time of radiolabelled peaks between test and control under HPLC analysis indicates that the drug under test is metabolized by S-mephenytoin 4'-hydroxylase activity. Often, the test is repeated using an extract from human liver in place of the 2C19 polypeptide. The appearance of a
35 labelled metabolic peak from the reaction using 2C19 recombinant organisms or 2C19 recombinant cell fractions having the same HPLC retention time, and a specific activity at least as high, as that observed for human liver microsomes

provides strong evidence that S-mephenytoin 4-hydroxylase activity plays a major role in processing the drug. The test can also be repeated using other 2C members, such as 2C18, as controls, in place of 2C19.

5 Drugs can also be screened for metabolic dependence on S-mephenytoin 4'-hydroxylase activity in transgenic nonhuman animals. Some such animals have genomes comprising a 2C19 transgene (e.g., SEQ. ID. No. 2) operably linked to control sequences so as to render the transgene capable of
10 being expressed in the animals. Other transgenic animals have a genome containing homozygous null mutations of endogenous 2C19 genes. Mice and other rodents are particular suitable for production of transgenic animals. Drugs are administered to transgenic animals in comparison with normal control
15 animals and the effects from administration are monitored. Drugs eliciting different responses in the transgenic animals than the control animals likely require S-mephenytoin 4'-hydroxylase activity for detoxification and/or activation.

 Drugs identified by the above screening methods as
20 being metabolized by S-mephenytoin 4'-hydroxylase activity should generally not be administered to individuals known to be deficient in this enzyme, or should be administered at different dosages. Indeed, in the absence of data on an individual patient's S-mephenytoin 4-hydroxylase phenotype, it
25 is often undesirable to administer such drugs to any member of an ethnic group known to be at high risk for S-mephenytoin 4-hydroxylase deficiency (e.g., Orientals and possibly blacks). If it is essential to administer drugs identified by the above screening procedures to individuals known to be at risk of
30 enzymic deficiency (e.g., no alternative drug is available), a treating physician is at least apprised of a need for vigilant monitoring of the patient's response to the drug. In general, the identification of a new drug as a substrate for 2C19 would mitigate against further development of the drug.

B. Screening Compounds for Mutagenic, Cytotoxic or Carcinogenic Activity

The invention provides methods of measuring the mutagenic, cytotoxic or carcinogenic potential of a compound.

5 In some methods, mutagenic, cytotoxic or carcinogenic effects are assayed directly on a cell line harboring one or more recombinant cytochrome P450 enzymes. In these methods, a compound under test is added to the growth medium of a cell line expressing 2C19, and/or 2C18 and/or other cytochrome
10 P450s. Often, one or more of the reagents discussed in Section V(I), *supra*, is also added. After a suitable incubation, mutagenic, cytotoxic or carcinogenic effects are assayed. Mutagenic effects are assayed, e.g., by detection of the appearance of drug-resistant mutant cell colonies
15 (Thompson, *Methods Enzymol.*, 58:308, 1979). For example, mutagenicity can be evaluated at the *hgp*rt locus (Penman et al., (1987) *Environ. Mol. Mutagenesis* 10, 35-60). Cytotoxicity can be assayed from viability of the cell line harboring the P450 enzyme(s). Carcinogenicity can be assessed
20 by determining whether the cell line harboring the P450 enzymes has acquired anchorage-independent growth or the capacity to induce tumors in athymic nude mice.

In other methods, a suspected compound is assayed in a selected test cell line rather than a cell line harboring
25 P450 enzymes. In these methods, the compound under test is contacted with P450 2C19 and/or 2C18 and/or other P450 enzymes. The P450 enzyme(s) can be provided in purified form, or as components of lysates or microsomal fractions of cells harboring the recombinant enzyme(s). The P450 enzyme(s) can
30 also be provided as components of intact cells. Usually, one or more of the reagents discussed in Section V(1), *supra*, is also added. Optionally, the appearance of metabolic products from the suspected compound can be monitored by techniques such as thin layer chromatography or high performance liquid
35 chromatography and the like.

The metabolic products resulting from treatment of the suspected compound with P450 enzyme(s) are assayed for mutagenic, cytotoxic or carcinogenic activity in a test cell

line. The test cell line can be present during the metabolic activation of the mutagen or can be added after activation has occurred. Suitable test cell lines include a mutant strain of *Salmonella typhimurium* bacteria having auxotrophic histidine mutations (Ames et al., *Mut. Res.* 31:347-364 (1975)). Other standard test cell lines include chinese hamster ovary cells (Galloway et al., *Environ. Mutagen.* 7:1 (1985); Gulati et al., (*Environ. Mol. Mutagenesis* 13:133-193 (1989)) for analysis of chromosome aberration and sister chromatic exchange induction, and mouse lymphoma cell (Myhr et al., *Prog. Mut. Res.* 5:555-568, (1985)).

The use of defined P450 enzymes for activation of compounds in the present methods offers significant advantages over previous methods in which rat or human S9-supernatant liver fractions (containing an assortment of P450 enzymes) were used. The present methods are more reproducible and also provide information on the mechanisms by which mutagenesis, cytotoxicity and carcinogenicity are effected.

20 C. Identification of Potential Chemopreventive Drugs

The invention also provides methods for identifying drugs having chemopreventive activity. These methods employ similar procedures to those discussed in paragraph (2) above except that the methods are performed using a known mutagenic, cytotoxic or carcinogenic agent, together with a suspected chemopreventive agent. Mutagenic, cytotoxic or carcinogenic effects in the presence of the chemopreventive agents are compared with those in control experiments in which the chemopreventive agent is omitted.

D. Screening for Potential Chemotherapeutic Drugs

The invention provides analogous methods to those described in paragraph (2), *supra*, for screening chemotherapeutic agents. In some methods, chemotherapeutic activity is determined directly on a tumorigenic cell line expressing 2C19 and/or 2C18 and or other cytochrome P450 enzymes. In other methods, chemotherapeutic activity is

determined on a tumorigenic test cell line. Chemotherapeutic activity is evidenced by reversion of the transformed phenotype of cells resulting in reduced 50bb agar growth or reduced tumor formation in nude mice.

5

E. Programmed Cell Death

The invention provides analogous methods to those described in paragraph (2), *supra*, for identifying agents that induce programmed cell death or apoptosis. Apoptosis may have an important impact on prevention of malignant transformation. Programmed cell death is assayed by DNA fragmentation or cell-surface antigen analysis.

10

F. Monitoring 2C18 and 2C19 Polypeptides

15

20

The invention provides methods of quantitating the amount of the specific protein in mammalian tissues by measuring the complex formed between the antibody and proteins in the tissue. For example, a biological sample is contacted with an antibody under conditions such that the antibody binds to specific proteins forming an antibody:protein complex which can be quantitatively detected.

VI. Diagnosing 2C19 and 2C18 Polymorphisms

Diagnostic Assays for Identifying Individuals Deficient in S-mephenytoin 4'-Hydroxylase

25

30

35

The invention provides a variety of assays for identifying individuals deficient in S-mephenytoin 4'-hydroxylase activity. Such individuals comprise about 3-5% of Caucasian populations and about 20% of Orientals and possibly blacks. Identification of individuals deficient in S-mephenytoin 4'-hydroxylase activity is important in selecting appropriate drugs for treatment of these individuals. Usually, drugs that are metabolized by S-mephenytoin 4'-hydroxylase should not be administered to these individuals. The assays diagnose mutations in cDNA or genomic DNA encoding 2C19, which as discussed above, is the principal human determinant of S-mephenytoin 4'-hydroxylase activity. The cDNA assays are particularly useful for *de novo* localization

of a 2C19 mutation to a particular nucleotide or nucleotides. The genomic assays are particularly useful for large-scale screening of individuals for the presence of a mutation that has previously been localized.

5

A. Amplification Technologies

Many of the diagnostic assays rely on amplification of part or all of a DNA segment encoding a 2C19 polypeptide (e.g., a 2C19 gene). In a preferred embodiment, target
10 segments encoding a 2C19 polypeptide are amplified by the polymerase chain reaction. The PCR process is described in e.g., U.S. Patent Nos. 4,683,195; 4,683,202; and 4,965,188; *PCR Technology: Principles and Applications for DNA Amplification* (ed. Erlich, Freeman Press, New York, NY, 1992);
15 *PCR Protocols: A Guide to Methods and Applications* (eds. Innis et al., Academic Press, San Diego, CA (1990); Mattila et al. *Nucleic Acids Res.* 19:4967 (1991); Eckert & Kunkel *PCR Methods and Applications* 1:17 (1991); *PCR* (eds. McPherson et al., IRL Press, Oxford) (each of which is incorporated by reference in
20 its entirety for all purposes). Reagents, apparatus and instructions for using the same are commercially available (e.g., from PECL). Other amplification systems include, ligase chain reaction, QB RNA replicase and RAN-transcription-based amplification systems.

25 To amplify a target nucleic acid sequence in a sample by PCR, the sequence must be accessible to the components of the amplification system. Accessibility can be achieved by isolating the nucleic acids from the sample. A variety of techniques for extracting nucleic acids from
30 biological samples are known in the art. Alternatively, if the sample is fairly readily disruptable, the nucleic acid need not be purified prior to amplification by the PCR technique, i.e., if the sample comprises cells, particularly peripheral blood lymphocytes or monocytes, lysis
35 and dispersion of the intracellular components may be accomplished merely by suspending the cells in hypotonic buffer. See Han et al. *Biochemistry* 26:1617-1625 (1987).

For amplification of mRNA sequences, a first step is the synthesis of a DNA copy (cDNA) of the region to be amplified by reverse transcription. Reverse transcription is the polymerization of deoxynucleoside triphosphates to form primer extension products that are complementary to a ribonucleic acid template. The process is effected by reverse transcriptase, an enzyme that initiates synthesis at the 3'-end of the primer and proceeds toward the 5'-end of the template until synthesis terminates. Examples of suitable polymerizing agents that convert the RNA target sequence into a complementary, copy-DNA (cDNA) sequence are avian myeloblastosis virus reverse transcriptase and *Thermus thermophilus* DNA polymerase, a thermostable DNA polymerase with reverse transcriptase activity marketed by PEFI. Reverse transcription can be carried out as a separate step, or in a homogeneous reverse transcription-polymerase chain reaction (RT-PCR). Polymerizing agents suitable for synthesizing a complementary, copy-DNA (cDNA) sequence from the RNA template are reverse transcriptase (RT), such as avian myeloblastosis virus RT, Moloney murine leukemia virus RT, or *Thermus thermophilus* (*Tth*) DNA polymerase, a thermostable DNA polymerase with reverse transcriptase activity marketed by PEFI.

The first step of each amplification cycle of the PCR involves the separation of the nucleic acid duplex formed by the primer extension. Strand separation is achieved by heating the reaction to a sufficiently high temperature for an sufficient time to cause the denaturation of the duplex but not to cause an irreversible denaturation of the polymerase (see U.S. Patent No. 4,965,188). Typical heat denaturation involves temperatures ranging from about 80°C to 105°C for times ranging from seconds to minutes. Typically, any initial RNA template is also degraded during the denaturation step leaving only DNA template. Other means of strand separation, including physical, chemical, or enzymatic means, are also possible.

Once the strands are separated, the next step involves hybridizing the separated strands with primers that

flank the target sequence. The primers are then extended to form complementary copies of the target strands. Template-dependent extension of primers in PCR is catalyzed by a polymerizing agent in the presence of adequate amounts of four deoxyribonucleotide triphosphates (typically dATP, dGTP, dCTP, and dTTP) in a reaction medium comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents include, for example, *E. coli* DNA polymerase I or its Klenow fragment, T_4 DNA polymerase, *Tth* polymerase, and *Taq* polymerase, a heat-stable DNA polymerase isolated from *Thermus aquaticus* commercially available from Perkin-Elmer Cetus Instruments (PECI, Norwalk, CT). See U.S. Patent No. 4,889,818. See Gelfand, 1989 in *PCR Technology*, *supra*. The polymerizing agents initiate synthesis at the 3'-end of the primer and proceeds toward the 5'-end of the template until synthesis terminates.

The primers are designed so that the position at which each primer hybridizes along a duplex sequence is such that an extension product synthesized from one primer, when separated from the template (complement), serves as a template for the extension of the other primer. The cycle of denaturation, hybridization, and extension is repeated as many times as necessary to obtain the desired amount of amplified nucleic acid.

The primers are selected to be substantially complementary to the different strands of each specific sequence to be amplified. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer with the remainder of the primer sequence being complementary to the strand. Alternatively, complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to be amplified to hybridize therewith and thereby form a

template for synthesis of the extension product of the other primer.

Paired primers for amplification of a given segment of DNA are designated forward and reverse primers.

5 Conventionally, the orientation of a double-stranded DNA molecules is that of the sense (or coding strand), with the 5'-terminus of the coding strand being drawn on the left (see, e.g., Fig. 15). Under this convention, the forward primer hybridizes to a double-stranded DNA molecule at a position 5'
10 (or upstream) from the reverse primer. The forward primer hybridizes to the complement of the coding strand of the double stranded sequence (*i.e.*, the antisense strand) and the reverse primer hybridizes to the coding strand.

The appropriate length of a primer depends on the
15 intended use of the primer but typically ranges from 10-100, 15-50, 15-30, or more usually, 15 to 25 nucleotides. Shorter primers tend to lack specificity for a target nucleic acid sequence and generally require cooler temperatures to form sufficiently stable hybrid complexes with the template.
20 Longer primers are expensive to produce and can sometime self-hybridize to form hairpin structures.

The spacing of primers determines the length of segment to be amplified. The spacing is not usually critical and amplified segments can range in size from about 25 bp to
25 at least 35 kbp. Segment from 25-2000, 50-1000, 100-500 bp or about 400 bp are typical. For larger segments, difficulties may occasionally be encountered in obtaining efficient and accurate amplification. For smaller segments, analysis of amplification products may be more difficult.

30 The primer can be labelled, if desired, by incorporating a label detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ³²P, fluorescent dyes, electron-dense reagents, enzymes (as commonly used in an
35 ELISA), biotin, or haptens and proteins for which antisera or monoclonal antibodies are available. A label can also be used to "capture" the primer, so as to facilitate the

immobilization of either the primer or a primer extension product, such as amplified DNA, on a solid support.

B. Tissue Sample for Analysis

5 The diagnostic assays are performed on a tissue sample containing a nucleic acid encoding a 2C19 polypeptide. For assay of genomic DNA, virtually any tissue sample (other than pure red blood cells) is suitable. For example, convenient tissue samples include whole blood, buccal, skin
10 and hair. For assay of cDNA, the tissue sample must be obtained from an organ in which a 2C19 gene is expressed, such as the liver. Liver samples from dead patients are suitable for *de novo* localization of mutations (see Section C, *infra*). However, for screening of living persons, liver biopsies,
15 while feasible, are generally undesirable. Thus, for large-scale screening of living persons, analysis of genomic DNA is preferred.

C. De Novo Localization of 2C19 Polymorphisms

20 2C19 polymorphisms are identified and localized to specific nucleotides by comparison of nucleic acids from poor metabolizing individuals with nucleic acids from extensive metabolizers. The comparison can be initiated directly at the genomic level. If intron primers are known, individual exons
25 and intron/exon junctions of 2C19 can be amplified from genomic DNA. These fragments can be sequenced directly or analyzed by single-stranded conformational analysis to indicate the presence of a polymorphism and then analyzed by sequencing.

30 Comparison is sometimes initiated at the cDNA level because of the shorter size of cDNA (about 1750 bp) relative to genomic DNA (about 55 kbp). cDNA is amplified from liver samples of individuals known to have phenotypic S-mephenytoin metabolic deficiencies, and the cDNA sequence is compared with
35 the wildtype sequence shown in SEQ. ID. No. 2. Often, the full-length cDNA is amplified. An initial comparison can be performed by single-stranded conformational analysis to indicate the existence of a polymorphism. The polymorphism is

then localized by sequence analysis indicating the site of mutations in cDNA. Of course, the amplification product can also be sequenced directly without prior conformational analysis. Having localized a mutation in cDNA, a
5 corresponding region of genomic 2C19 DNA is amplified. The genomic DNA is usually amplified from primers spanning the mutation. At least one of the primers for this amplification usually comprises a subsequence of the cDNA sequence proximate (i.e., within 25-200 bp of the cDNA mutation). Primers can
10 also comprise subsequences of genomic 2C19 DNA that have already been sequenced, subsequences from related genomic sequences, such as 2C18 or 2C9 (see de Morais et al., *Biochem. Biophys. Res. Commun.* 194:194-201 (1993)) (incorporated by reference in its entirety for all purposes), or can be random.
15 An amplified genomic fragment spanning the portion of the coding region in which the cDNA polymorphism occurs is sequenced and compared with the corresponding region from a 2C19 sequence from an individual exhibiting extensive S-mephenytoin 4'-hydroxylase metabolism to identify the locus of
20 the genomic mutation.

In some instances, there will be a simple relationship between genomic and cDNA mutations. That is, a single base change in a coding region of genomic DNA can give rise to a corresponding mutated codon in the cDNA. In other
25 instances, the relationship between genomic and cDNA mutations is more complex. Thus, for example, a single base change in genomic DNA creating an aberrant splice site can give rise to deletion of a substantial segment of cDNA in a poor metabolizing individual.

30

D. The 681 and 636 Polymorphisms

The principal mutation in individuals deficient in the S-mephenytoin 4'-hydroxylase activity is designated the 681 polymorphism. See Example 7. The 681 polymorphism
35 results from a single-base mutation in genomic 2C19 DNA at nucleotide position 681 of the coding region. A nucleotide in a coding (i.e., exonic) region of genomic 2C19 DNA is designated the same number as the corresponding nucleotide in

the cDNA sequence shown in SEQ. ID. No. 2, when the genomic coding sequence is maximally aligned with the cDNA sequence. The 681 polymorphism results in a G/A transposition at nucleotide 681 of the coding region. Homozygous mutations at this position occur in about 70% of individuals having a low-producing (*i.e.*, defective) S-mephenytoin 4'-hydroxylase phenotype. The mutation is inherited in an autosomal recessive fashion. Thus, individuals heterozygous in this mutation usually exhibit normal (*i.e.*, extensive S-mephenytoin activity). Fortuitously, the mutation confers two distinct properties that facilitate its identification. In genomic DNA, the polymorphism results in loss of several restriction enzyme sites (e.g., SmaI) and acquisition of other restriction sites (e.g., EcoRII) site in mutant individuals compared with wildtype individuals. These restriction sites include the 681 nucleotide. In mRNA or cDNA, the 681 mutation results in a deletion of 40 bp spanning nucleotides 643-682 of the wildtype cDNA sequence shown in Fig. 12. The deletion is the consequence of an altered splice pattern stemming from the presence of the 681 polymorphism in genomic DNA.

A second polymorphism is designated the 636 polymorphism. See Example 8. The 636 polymorphism results from a single-base mutation in genomic 2C19 DNA at nucleotide position 636. The 636 polymorphism results in a G/A transposition thereby introducing a premature stop codon into 2C19 mRNA. The mutation is easily be recognized by the loss of e.g., a BamHI site in both genomic and cDNA and acquisition of e.g., a HinfI site. The mutation is inherited in an autosomal recessive fashion. Homozygous mutations at nucleotide 636 account for about 10% of low-producing phenotypes in Orientals. Heterozygous individuals having one allele defective in the 636 polymorphism and the other allele defective in the 681 polymorphism account for all or nearly all of the remaining 15% of low producing Oriental individuals. Thus, the 681 and 636 polymorphisms collectively account for all, or nearly all, low producing phenotypes in Orientals.

In Caucasians, the 636 polymorphism is less prevalent and some low producing individuals probably have a mutation at a locus other than nucleotide 681 or 636 of the coding sequence. Conceivably, a few mutations might occur in other genes that exert regulatory control over the 2C19 gene. However, most, if not all, of the remaining mutations probably result from additional polymorphisms in the 2C19 gene.

E. Screening Assays for Defined Mutations

The invention provides assays that permit large-scale screening of individuals for the presence of defined mutations. Of course, detection of the 681 and 636 mutations, which account for all or nearly all deficiencies in Orientals and about 75% of deficiencies in Caucasians, is of primary importance. An assay on an individual under test is often performed in parallel with control assays on DNA samples from subjects of known phenotype (i.e., extensive or poor metabolizer of S-mephenytoin).

1. Genomic Assays

Assays are preferably performed on a genomic substrate because of the ready availability of tissue samples containing genomic DNA.

a. Amplification of Segments Spanning a Defined Mutation

A preferred strategy for analysis entails amplification of a DNA sequence spanning previously localized polymorphism(s) (e.g., the 681 and/or 636 polymorphisms). Amplification of such a sequence can be primed from forward and reverse primers that hybridize to a 2C19 gene on opposite sides of a mutation (e.g., the 681 mutation, but which do not hybridize to the mutated nucleotide itself). That is, for detection of the 681 polymorphism, the forward primer hybridizes upstream or 5' to the 681 nucleotide and the reverse primer hybridizes downstream or 3' to this nucleotide. Similarly, for detection of the 636 polymorphism, the forward primer hybridizes upstream or 5' to the 636 nucleotide and the

reverse primer hybridizes downstream or 3' to this nucleotide. For simultaneous analysis of 636 and 681 polymorphisms, the forward primer hybridizes upstream or 5' to the 636 nucleotide and the reverse primer hybridizes downstream or 3' to nucleotide 681.

The forward primer is sufficiently complementary to the antisense strand of a 2C19 DNA sequence to hybridize therewith and the reverse primer is sufficiently complementary to the sense strand of the 2C19 sequence to hybridize therewith. The primers usually comprise first and second subsequences from opposite strands of a double-stranded 2C19 DNA sequence. Isolated points of mismatch between a primer and a corresponding 2C19 subsequence can usually be tolerated but are not preferred. It is particularly important to avoid mismatches in the two nucleotides at the 3' end of the primer (especially the terminal nucleotide).

Because allelic variants of 2C19 exhibit at least about 97% sequence identity to each other, it is not critical which variant is selected as a source of subsequences for incorporation into forward and reverse primers. For example, suitable subsequences can be obtained from the genomic 2C19 sequence defined as wildtype in Figs. 15-17. Fig. 15 provides genomic sequence immediately flanking the 681 mutation, and Figure 16 provides more distal flanking sequences. Figure 17 provides genomic sequence flanking the 636 mutation. These figures provide sufficient sequence for selection of a multitude of paired primers for amplification of a sequence spanning the 681 and/or 636 polymorphisms. Although there is no apparent advantage for doing so, additional genomic sequence flanking the regions already sequenced could easily be determined by PCR-based gene walking. See Parker et al., *Nucl. Acids Res.* 19:3055-3060. A specific primer for the sequenced region is primed with a general primer that hybridizes to the flanking region.

Forward primers often comprise about 10-50 and preferably 15-30 contiguous nucleotides from the wildtype 2C19 sequences shown in Figs. 15-17 (which is the coding or sense sequence). Reverse primers often comprise about 10-50 or 15-

30 nucleotides from the complement of the wildtype 2C19 sequence shown in Figs. 15-17. The complement of the sequence shown in Figs. 15-17 is also referred to as the antisense sequences. A primer (or its complement) preferably exhibits 100% sequence identity with a corresponding 2C19 subsequence to which it hybridizes over a window of about 15-30 bp. For amplification of the 681 polymorphism, forward primers preferably comprise a segment of contiguous nucleotides from the fourth intronic region and reverse primers a segment of contiguous nucleotides from the fifth exonic or intronic region. For amplification of the 636 polymorphism, forward primers preferably comprise a segment of contiguous nucleotides from the third intronic region and reverse primers a segment of contiguous nucleotides from the fourth intronic region. For amplification of both the 636 and 681 polymorphisms, forward primers preferably comprise a segment of contiguous nucleotides from the third intronic region and reverse primers a segment of contiguous nucleotides from the fifth exonic region or fifth intronic region. See Figure 19. As noted above, the spacing of the subsequences is not critical, but a separation of about 50-2000 bp. For simultaneous amplification of the 636 and 681 mutations, the spacing is typically 1000-1500 bp. For amplification of either mutation alone, a spacing of about 400 bp is typical.

Preferred primers exhibit perfect sequence identity to 2C19 and lesser sequence identity to corresponding regions of related genes, such as 2C9 and 2C18. Such primers are designed by comparison of the wildtype 2C19 sequence shown in Fig. 15-17 with corresponding sequences from 2C9 and 2C18 described by de Moraes et al., *supra*. In general, sequence divergence between the three genes is expected to be greater in intronic sequences. An exemplary pair of primers for amplifying a segment spanning the 681 mutation is described in Example 7. A forward primer, 5'-AATTACAACCAGAGCTTGGC-3' (SEQ. ID. No. 55), exhibits perfect sequence identity to a subsequence from the wildtype 2C19 sense strand within intron 4. A reverse primer 5'-TATCACTTTCCATAAAAGCAAG-3' ((SEQ. ID. No. 56) exhibits perfect sequence identity to the

antisense strand of the wildtype 2C19 sequence within exon 5. The amplification product from these primers has a length of 169 bp. An exemplary pair of primers for amplifying a segment spanning the 636 mutation is described in Example 8. A

5 forward primer, 5'-TATTATCTGTAACTAATATGA-3' (SEQ. ID. No. 57) exhibits perfect sequence identity to a subsequence from the wildtype 2C19 sense strand within intron 3. A reverse primer 5'-ACTTCAGGGCTTGGTCAATA-3' (SEQ. ID. No. 58) exhibits perfect
10 sequence identity to the antisense strand of the wildtype 2C19 sequence within intron 4. The amplification product from these primers has a length of 329 bp.

Having amplified a segment of a 2C19 gene known to span a polymorphism, a variety of assays are available for determining whether a mutation is present in an individual
15 under test. A generally applicable, but relatively laborious assay, is to sequence the amplified fragment across the polymorphic locus and compare the resulting sequence with the wildtype 2C19 sequence shown in Fig. 15-17.

A simpler assay, but one applicable to only certain
20 mutations, is to compare the size or restriction profile of the amplified segment, optionally in comparison with a corresponding wildtype 2C19 segment. For the 681 polymorphism, restriction analysis provides a rapid and clear-cut means of identifying a mutant allele. The 681
25 polymorphism results in loss of a SmaI site and acquisition of an EcoRII site in mutant alleles. Thus, SmaI digestion of a wildtype allele produces an extra band compared with a mutant allele. For the amplification product obtained using the exemplified primers discussed above, SmaI digestion of the
30 wildtype product yields fragments of 120 and 49 bp, whereas the mutant amplification product remains uncut yielding a single fragment of 169 bp. In individuals homozygous for the wildtype allele, only the 120 bp and 49 bp bands are present. In individuals homozygous for the mutant allele, only the 169
35 bp band is present. In heterozygotes, all three bands (*i.e.*, 169, 120 and 49 bp) are present. The bands can usually be detected by agarose or acrylamide gel electrophoresis and ethidium bromide staining. If greater sensitivity is needed,

the amplification product is labelled and the bands detected by, e.g., autoradiography. Of course, the assay can also be performed using an isoschizomer of SmaI with identical results. The assay can also be performed by digesting with
5 EcoRII or an isoschizomer thereof. In this case, one obtains a mirror image of the results obtained for SmaI digestion, because the mutant 2C19 allele contains an additional EcoRII site relative to the wildtype allele. As a quality control measure, both SmaI and EcoRII digestions can be performed on
10 separate aliquots of a test sample. Of course, any other enzyme that recognizes a site that includes the 681 polymorphism can also be used. For example, alternatives to SmaI (i.e., that cleave only the wildtype allele) include AvaI, MspI, NciI, ScrFI and TspEI).

15 The 636 polymorphism can be similarly analyzed by digestion with e.g., BamHI. BamHI digestion of a wildtype allele produces an extra band compared with a mutant allele. For the amplification product obtained using the exemplified primers discussed above, BamHI digestion of the wildtype
20 product yields fragments of 233 and 96 bp, and digestion of the mutant product yields a single fragment of 329 bp. In individuals homozygous for the wildtype allele, only the 233 bp and 96 bp bands are present. In individuals homozygous for the mutant allele, only the 329 band is present. In
25 heterozygotes, all three bands are present. Of course, other enzymes that cut the wildtype allele at the polymorphic locus but not the 636 mutant allele, or vice versa, can also be used. For example, alternatives to BamHI include AlwI, BsaJI, BstVI, DpnI, EcoRII, NlaIV, Sau3AI and ScrFI. Enzymes that
30 recognize a site on the mutant allele including nucleotide 636, but do not recognize the wildtype allele, include HinfI and TfiI.

For simultaneous detection of the 681 and 636 polymorphisms after amplification of a fragment spanning both
35 polymorphism, the DNA can be double digested with two of the enzymes mentioned above. One enzyme should distinguish between the mutant 681 allele from a wildtype allele and the other should distinguish the mutant 636 allele from a wildtype

allele. For example, double digestion with SmaI and BamHI is suitable. The double digestion generates six different restriction patterns corresponding to the six possible genotypes: wt/wt, wt/681, wt/636, 681/681, 636/636 and 681/636. See Figure 19.

In another assay, amplification products are subjected to single-stranded conformational analysis. See, e.g., Hayashi, *PCR Methods & Applications* 1, 34-38 (1991); Orita, *Proc. Natl. Acad. Sci. USA* 86, 2766-2270 (1989); Orita et al., *Genomics* 5, 874-879 (1989). This method is capable of detecting many single base mutations in DNA fragments up to 200 bp irrespective whether the mutation causes a change in restriction fragment profile. In this method, the PCR reaction is performed using at least one labelled nucleotide or labelled primer to obtain a labelled amplified fragment. The amplification product is then denatured and the strands resolved by polyacrylamide gel electrophoresis under nondenaturing conditions. Mutations are detected by altered mobility of separated single strands.

b. Selective Amplification of an Allelic Variant

An alternative method for detecting defined mutations in a 2C19 gene employs a selective strategy whereby a wildtype allele is amplified without amplification of a mutant allele (or vice versa). This is accomplished by designing one of the primers to hybridize to a subsequence overlapping a defined polymorphism (for example, the 681 polymorphism). Such a primer can be designed to hybridize to one polymorphic allele without hybridizing to the other. Thus, when such a primer is paired with a second primer hybridizing distal to the polymorphic region, amplification will only occur for one polymorphic allele.

For diagnosis of the 681 polymorphism, selective amplification of the wildtype allele of 2C19 can be accomplished using a forward primer that has about 10-50, and usually 15-30 nucleotides from the wildtype 2C19 sequence shown in Fig. 15 or 16, including nucleotide 681. Such a

forward primer when paired with any suitable reverse primer downstream from nucleotide 681 (i.e., sufficiently complementary to the sense strand of 2C19 to hybridize therewith) can be used to amplify selectively the wildtype allele without amplifying a mutant allele. The selectivity between amplification of wildtype and mutant alleles is greatest when the 681 nucleotide occurs near, or preferably, at the 3' end of the primer. Because the extension forms from the 3' end of the primer, a mismatch at or near this position is most inhibitory of amplification. The same result can be achieved by using a reverse primer that has about 10-50 or usually 15-30 contiguous nucleotides from the complement of the wildtype 2C19 sequence shown in Fig. 15 or 16 (i.e., the antisense strand) including the nucleotide at position 681. Such a reverse primer can be paired with any suitable forward primer sufficiently complementary to a subsequence of the antisense strand of the 2C19 gene upstream from nucleotide 681 to hybridize therewith. The 681 nucleotide should again be at or near the 3' end of the reverse primer.

Selective amplification of a 681 mutant allele is accomplished by an analogous strategy in which primers are designed to hybridize to the mutant allele without hybridizing to the wildtype. A suitable forward primer for amplification comprises about 10-50 or usually 15-30 contiguous nucleotides from the mutant 2C19 sequence shown in Fig. 15 or 16 (i.e., the sense strand). The forward primer can be paired with any suitable reverse primer sufficiently complementary to the sense strand of a downstream 2C19 subsequence to hybridize therewith. Alternatively, the same result can be achieved using a reverse primer comprising about 10-50 or 15-30 contiguous nucleotides from the complement of the mutant 2C19 sequence shown in Fig. 15 or 16 (i.e., the antisense strand). Such a reverse primer can be paired with any suitable forward primer sufficiently complementary to the antisense strand of an upstream 2C19 subsequence to hybridize therewith.

For diagnosis of the 636 polymorphism, selective amplification of the wildtype allele of the 2C19 allele can be accomplished using a forward primer that has about 10-50, and

usually 15-30 nucleotides from the wildtype 2C19 genomic sequence shown in Fig. 17, including nucleotide 636. Such a forward primer when paired with any suitable reverse primer downstream from nucleotide 636 (*i.e.*, sufficiently
5 complementary to the sense strand of 2C19 to hybridize therewith) can be used to amplify selectively the wildtype allele without amplifying a mutant allele. The 636 nucleotide usually occurs near, or preferably, at the 3' end of the primer. The same result can be achieved by using a reverse
10 primer that has about 10-50 or usually 15-30 contiguous nucleotides from the complement of the wildtype 2C19 genomic sequence shown in Fig. 17 (*i.e.*, the antisense strand) including the nucleotide at position 636. Such a reverse primer can be paired with any suitable forward primer
15 sufficiently complementary to a sequence of the antisense strand of the 2C19 gene upstream from nucleotide 636 to hybridize therewith. The 636 nucleotide should again be at or near the 3' end of the reverse primer.

For selective amplification of a 636 mutant allele a
20 suitable forward primer for amplification comprises about 10-50 or usually 15-30 contiguous nucleotides including nucleotide 636 from the mutant 2C19 genomic sequence shown in Fig. 17 (*i.e.*, the sense strand). The forward primer can be paired with any suitable reverse primer sufficiently
25 complementary to the sense strand of a 2C19 genomic subsequence downstream from nucleotide 636 to hybridize therewith. Alternatively, the same result can be achieved using a reverse primer comprising about 10-50 or 15-30 contiguous nucleotides including nucleotide 636 from the
30 complement of the mutant 2C19 sequence shown in Fig. 17 (*i.e.*, the antisense strand). Such a reverse primer can be paired with any suitable forward primer sufficiently complementary to the antisense strand of a 2C19 subsequence upstream from nucleotide 636 to hybridize therewith.

35 Following amplification, the sample under test is characterized as wildtype or mutant by the presence or absence of an amplification product. With a primer designed for selective amplification of the wildtype allele, the presence

of an amplification product is indicative of that allele and the absence of an amplification product indicative of a mutant allele. The converse applies for primers designed for selective amplification of a mutant allele. In preferred
5 assay, a sample is divided into two aliquots, one of which is amplified using primers for wildtype allele amplification, the other of which is amplified using primers appropriate for mutant allele amplification. The presence of an amplification product in one but not both of the aliquots indicates that the
10 individual under test is either wildtype or a homozygous for the mutation (depending on aliquot in which the amplification product occurred). The presence of amplification product in both aliquots indicates that the individual is heterozygous. The absence of an amplification product in both aliquots would
15 indicate either the absence of a 2C19 gene or a quality control problem in the amplification procedure requiring that the assay be repeated. Coamplification of a second known standard human gene using a second set of primers can aid in distinguishing between these possibilities. If both bands are
20 missing, the problem is probably quality control, while amplification of only the standard gene is suggestive that the CYP2C19 gene may be deleted.

The presence or absence of amplification products can be detected by gel electrophoresis. Gels are usually
25 visualized by ethidium bromide staining. However, if greater sensitivity is required fragments can be labelled in the course of amplification. Amplified fragments can be electrophoresed directly or can be cut with any restriction enzyme that releases fragments of a convenient size from the
30 amplification products. For the simultaneous analysis of multiple samples, the dot-blot method may be advantageous. In the dot blot method, multiple unlabelled amplification mixtures are bound to discrete locations on a solid support, such as a membrane. The membrane is incubated with labeled
35 probe under suitable hybridization conditions, the unhybridized probe removed by washing, and the filter monitored for the presence of bound probe.

c. Southern Blotting

For polymorphic mutations resulting in loss or acquisition of a restriction site (such as the 681 and 636 polymorphisms), samples of genomic DNA can also be analyzed by Southern blotting without the need for prior amplification. The DNA is digested with an enzyme that cuts a wildtype allele but not a mutant allele or vice versa (e.g., BamHI, SmaI, EcoRII or HinfI, or isoschizomers of any of these). For analysis of the 681 polymorphism, digestion with SmaI or isoschizomers results in an additional fragment from the wildtype allele compared with the mutant allele. Digestion with EcoRII or isoschizomers results in an additional fragment from the mutant allele. Digestion products are detected with a 2C19 probe. For analysis of the 636 polymorphism, digestion with BamHI or isoschizomers results in an additional fragment from the wildtype allele compared with the mutant allele. Digestion with HinfI results in an additional fragment from the mutant allele. The probe can be any segment of a 2C19 DNA sequence that includes the polymorphism and extends for at least about 20 nucleotides on either side.

2. cDNA Assays

Defined polymorphisms can also be detected by analysis of cDNA by similar strategies to those employed for genomic DNA. However, the primers appropriate for amplification procedures are not necessarily interchangeable for the two substrates. Suitable primers for analysis of the 681 and 636 polymorphisms in cDNA are described below.

a. Amplification of Segments Spanning a Defined Mutation

The 681 polymorphism in genomic DNA results in a 40 bp deletion of cDNA comprising nucleotides 643-682 of the wildtype 2C19 cDNA or genomic sequence shown in Fig. 12. The forward primer and reverse primers are therefore designed to hybridize to 2C19 subsequences on opposite sides of this deletion. Thus, for example, a forward primer can hybridize to the antisense strand of a 2C19 sequence upstream from

nucleotide 643 of the coding region. Such a forward primer should be paired with a reverse primer that hybridizes to the sense strand of the 2C19 sequence downstream from nucleotide 682. Nucleotides in a 2C19 DNA sequence are designated the numbers of corresponding nucleotides in the wildtype cDNA sequence shown in SEQ. ID. No. 2 (or Fig. 12, which shows a subsequence of SEQ. ID. No. 2), when the sequences are maximally aligned. Preferably, the forward primer comprises about 10-50 or 15-30 contiguous nucleotides upstream of nucleotide 645 from the wildtype 2C19 cDNA sequence shown in Fig. 12 or SEQ. ID. No. 2. Analogously, the reverse primer preferably comprises about 10-50 or 15-30 contiguous nucleotides from the complement of the wildtype 2C19 cDNA sequence shown in Fig. 12 or SEQ. ID. No. 2 downstream from nucleotide 682 of the coding region. For example, a forward primer comprising 5'-ATTGAATGAAAACATCAGGATTG-3' (SEQ. ID. No. 59) and a reverse primer comprising 5'-GTAAGTCAGCTGCAGTGATTA-3' (SEQ. ID. No. 60) form a suitable pair. The amplification product from such primers is 40 bp longer for the wildtype 2C19 cDNA sequence than for the 681 mutant sequence.

For detection of the 636 polymorphism, the forward primer and reverse primers are designed to hybridize to 2C19 subsequences on opposite sides of nucleotide 636. Thus, for example, a forward primer can hybridize to the antisense strand of a 2C19 sequence upstream from nucleotide 636 of the coding region. Such a forward primer should be paired with a reverse primer that hybridizes to the sense strand of the 2C19 sequence downstream from nucleotide 636 (SEQ. ID. No. 2 or Fig. 12). Preferably, the forward primer comprises about 10-50 or 15-30 contiguous nucleotides upstream of nucleotide 636 from the wildtype 2C19 cDNA sequence shown in Fig. 12 or SEQ. ID. No. 2. Analogously, the reverse primer preferably comprises about 10-50 or 15-30 contiguous nucleotides from the complement of the wildtype 2C19 cDNA sequence shown in Fig. 12 or SEQ. ID. No. 2 downstream from nucleotide 636 of the coding region.

For simultaneous detection of the 636 and 681 polymorphisms, the forward primer should be as described for detection of the 636 polymorphism and the reverse primer as described for detection of the 681 polymorphism. These primers will amplify a segment of DNA spanning both the 636 and 681 polymorphisms.

Amplification products are usually analyzed by gel electrophoresis. The products can be analyzed uncut or can be cleaved with any restriction enzyme having a site in the amplification product. For detection of the 681 polymorphism, SmaI and its isoschizomers are particularly useful because of the presence of a restriction site present in wildtype 2C19 DNA that is not present in the mutant form. See Fig. 12. Similarly, BamHI and its isoschizomers are particularly useful for detection of the 636 polymorphism. Analysis of fragments allows distinction between wildtype, homozygous and heterozygous mutations as discussed for the corresponding genomic assay.

b. Selective Amplification of an Allelic Variant

For analysis of the 681 polymorphism, selective amplification of the wildtype variant is achieved by selecting a forward or reverse primer that overlaps nucleotides 643-682 of the wildtype 2C19 cDNA sequence (Fig. 12). This segment of nucleotides is not present in a mutant allele. Thus, a primer hybridizing to this segment of the wildtype allele will not hybridize to the mutant allele. Accordingly, such primers can be used to prime amplification of the wildtype allele without priming amplification of the mutant allele. For example, a forward primer that hybridizes to the complement of the wildtype 2C19 cDNA sequence shown in Fig. 12 between nucleotides 643-682 without hybridizing to the complement of the mutant 2C19 DNA sequence shown in Fig. 12 is suitable. Such a forward primer can be paired with any suitable reverse primer sufficiently complementary with a downstream subsequence of the sense strand of the 2C19 cDNA to hybridize therewith.

Alternatively, a reverse primer is designed that hybridizes to the wildtype 2C19 cDNA sequence shown in Fig. 12 between nucleotides 643 and 682 without hybridizing to the mutant 2C19 cDNA sequence shown in Fig. 12. Such a reverse primer can be paired with any suitable forward primer sufficiently complementary with an upstream subsequence of the antisense strand of the 2C19 cDNA to hybridize therewith.

Primers for selective amplification of the mutant allele can also be designed. A suitable primer hybridizes to two 2C19 subsequences, of about 1-50, 5-30 or 10-20 nucleotides, which subsequences are separated by nucleotides 643-682 in the wildtype sequence, but which are contiguous in the mutant sequence. Such primers hybridize to mutant 2C19 cDNA sequences without hybridizing to wildtype sequences. For example, a forward primer comprising a subsequence of nucleotides 633-642 of the wildtype 2C19 cDNA sequence shown in Fig. 12 joined to a second subsequence of nucleotides 684-693 of this sequence is suitable. This primer can be paired with any suitable reverse primer sufficiently complementary to a downstream subsequence of the sense strand of the 2C19 cDNA to hybridize therewith.

For analysis of the 636 polymorphism, primers can be designed using the same strategy as discussed for selective amplification of genomic DNA except that the primers, which include nucleotide 636, are formed from nucleotide segments from cDNA rather than genomic sequences.

Amplification products are analyzed using the same methods as described for corresponding genomic amplification products.

F. Diagnostic Kits

The invention also provides kits comprising useful components for practicing the diagnostic methods of the invention. The kits comprise at least one of the primers discussed above. Kits usually contain a matched pair of forward and reverse primers as described above for amplifying a segment encompassing the 681 and/or the 636 polymorphism. Some kits contain two matched pairs of primers, e.g., one pair

for analysis of the 681 polymorphism, the other pair for analysis of the 636 polymorphism. For selective amplification of mutant or wildtype alleles, kits usually contain a pair of primers for amplification of the mutant allele and/or a
5 separate pair of primers for amplification of the wildtype allele. Optional additional components of the kit include, for example, restriction enzymes for analysis of amplification products, such as BamHI, SmaI, HinfI and/or EcoRII (or isoschizomers of any of these), reverse-transcriptase or
10 polymerase, the substrate nucleoside triphosphates, means used to label (for example, an avidin-enzyme conjugate and enzyme substrate and chromogen if the label is biotin), and the appropriate buffers for reverse transcription, PCR, or hybridization reactions. Usually, the kit also contains
15 instructions for carrying out the methods.

G. Nucleic Acid Fragments

In another aspect, the invention provides fragments of a mutant 2C19 allele spanning the 681 polymorphism and/or
20 636 polymorphism. The fragments usually have up to about 50, 100, 200, 500, 1000, 2000 or 10,000 bp of 2C19 sequence. Some fragments comprise at least about ten contiguous nucleotides including nucleotide 681 from the mutant 2C19 allele shown in Fig. 15. Other fragments comprise at least about ten
25 contiguous nucleotides including nucleotide 636 from the mutant 2C19 allele shown in Fig. 17. The fragments can be single or double stranded. The fragments are provided in substantially purified form. Usually, the fragments are the result of PCR amplification. The fragments are useful in the
30 diagnostic assays discussed above.

The following examples are provided to illustrate but not to limit the invention.

35

EXAMPLES

Materials. Human liver samples were obtained from organ donors through the National Disease Research Interchange in Philadelphia, PA, and from the Human Liver Research

Facility, Stanford Research Institute, Life Sciences Division, Menlo Park, CA. Restriction endonucleases were purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). [α -³³] dCTP (3000 Ci/mmol) and [γ -³²P] ATP (500 Ci/mmol) and [α -³²S] dATP (650 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). All other reagents were of the highest quality available.

Conditions. Hybridization and washing conditions for screening libraries with random-labeled cDNAs for 2C13(g) or 254c used the same solutions as described for actin, but were performed at nonstringent temperatures (42°C). Conditions for hybridization of clones with T300R were identical with those described above. Hybridization of cDNA clones with M300R (recognizes 2C9, 2C10, and 2C19) (5'-ACTTTTCAATGTAAGCAAAT-3') (SEQ. ID. No. 17) was identical except that for each oligomer the hybridization temperature and the high-stringency wash were 5°C below the calculated melting temperatures.

Example 1: Construction and Screening of Human Liver cDNA Libraries

Two cDNA libraries were constructed from human livers 860624 and S33, which differed phenotypically in the hepatic content of P450 HLx (2C8) (SEQ. ID. No. 8). Several partial cDNA clones were found but no full-length clones.

A second cDNA library (from a liver phenotypically high in HLx) was then screened. Eighty-three essentially full-length (>1.8 kb) clones belonging to the 2C subfamily were isolated from this library. These include full-length clones for two additional new members of the 2C subfamily.

The majority of the cDNAs characterized in the high-HLx library (60%) were one of two allelic variants of 2C9, while 35% represented 2C8 (SEQ. ID. No. 8). Two new genes were identified (two allelic variants of 2C18 and 2C19).

The two cDNA libraries from individuals phenotypically high and low in HLx were examined to determine whether a variant mRNA for 2C8 (SEQ. ID. No. 8) was responsible for the polymorphic expression of HLx and to

identify additional members of the 2C subfamily. No clones for 2C8 (SEQ. ID. No. 8) were isolated from the individual phenotypically high individual. Two allelic variants for 2C9 were isolated. In addition, full-length cDNAs for two additional new members (2C18 and 2C19) were isolated. These new members of the 2C subfamily were expressed in COS-1 cells and shown to be immunochemically distinct from HLx and 2C9, and 2C18 metabolized racemic mephenytoin.

Total human liver RNA was prepared by the guanidine hydrochloride method (Cox, *Methods Enzymol.* 12:120-129 (1968)) from two human livers either low (860624) or high (S33) in HLx as identified by immunoblot analysis. Poly(A+)RNA was then isolated by two passages over an oligo(dT)-cellulose column (Aviv et al., *Proc. Natl. Acad. Sci. U.S.A.* 69:1408-1412 (1972)). The low-HLx cDNA library was prepared by Stratagene Cloning systems (La Jolla, CA), and the double-stranded cDNA was treated with S1 nuclease. Following the addition of EcoRI linkers, the double-stranded cDNA was size-fractionated on a CL-4B Sepharose column. The largest fraction was ligated into λ ZAPII and then transfected into XL1-Blue. The high-HLx cDNA library was constructed following the methods of Watson et al., in *DNA Cloning* (Glover, D.M., Ed.) 1:79-88, IRL Press, Washington, D.C. (1985)). Double-stranded cDNA was ligated to EcoRI linkers, size-fractionated on an agarose gel (1.8-2.4 kb), and then ligated into λ ZAPII (Stratagene) and transfected into XL1-Blue.

The low-HLx library was screened under conditions of low stringency with a 32 P-labeled rat P450 2C13 cDNA probe and with oligonucleotides for human 2C8 (SEQ. ID. No. 8) (T300R) (5'-TTAGTAATTCTTTGAGATAT-3') (SEQ. ID. No. 18) and 2C9 (M300R) (5'-CTGTTAGCTCTTTCAGCCAG-3') (SEQ. ID. No. 19). The high-HLx library was screened under conditions of low stringency using a 32 P-labeled 254C cDNA probe derived from the first library and M300R (2C9). Positive clones were isolated, transfected into XL1-Blue, and excised into the plasmid Bluescript, according to Stratagene's excision protocol.

Screening the cDNA library constructed from a low-HLx individual with a cDNA for rat 2C13 under nonstringent

conditions and with oligonucleotide probes specific for 2C8 (SEQ. ID. No. 8) and 2C9 yielded several clones for 2C9 and a partial DNA, clone 254c, which now appears to be an incompletely characterized splice variant of the P450 2C subfamily. None of the clones identified in this library were full-length. Clone 186 was identical with but 25 base pairs longer than MP-4, a 2C9 clone previously described by Ged et al. (1988).

Approximately 40000 plaques were then screened from the library from liver S33 with the cDNA for 254c under non-stringent conditions and with an oligonucleotide probe specific for 2C9. Eighty-three essentially full-length 2C clones (>1.8 kb) were isolated, purified, and partially or completely sequenced (Table I). Of these, 29 clones were found to encode cytochrome P450 2C8 (SEQ. ID. No. 8). One clone (7b) of 2C8 (SEQ. ID. No. 8) was isolated which was similar to Hpl-1 and Hpl-2 reported by Okino et al. (1987), but different by having a tyrosine at position 130 instead of an asparagine and an isoleucine at 264 instead of a methionine.

TABLE I
Distribution of P450 2C cDNA Clones from
Human Liver S33*

	No. of Clones	% Distribution
5	-----	-----
2C8 (SEQ. ID. No.8)	29	35
2C9		
65 (SEQ. ID. No. 10)	39	47
25 (SEQ. ID. No. 4)	11	13
10 2C10	0	0
2C18		
29c (SEQ. ID. No. 6)	1	1.2
6b (SEQ ID. No. 12)	2	2.5
2C19 (11A) (SEQ ID No. 2)	1	1.2
15 Total	83	100

* Clones were classified by hybridization with specific oligonucleotide probes and partial sequencing.

20 There are a number of polymorphisms in the human
CYP2C subfamily. These include variations in the hepatic
levels of HLx (Wrighton et al., *Arch. Biochem. Biophys.*
306:240-245 (1987)) and metabolic variations in the hepatic
metabolism of S-mephenytoin. The molecular basis for these
25 polymorphisms has not been characterized. 2C8 (SEQ. ID. No.
8) appears to encode the protein for HLx on the basis of its
N-terminal amino acid sequence (Okino et al., *J. Biol. Chem.*
262:16072-16079 (1987); Wrighton et al., *supra*; Lasker et al.,
Biochem. Biophys. Res. Commun. 148:232-238 (1987)).

30

Example 2: Sequence Analysis

35 The Bluescript plasmids containing the positive cDNA
inserts from the low-HLx library were purified by CsCl
gradients, while the plasmids containing cDNA inserts from the
high-HLx library were purified by using Qiagen plasmid
purification kits (Qiagen, Inc., Studio city, CA). The
double-stranded cDNA inserts were sequenced by the dideoxy
chain termination method reported in Sanger et al., *J. Mol.*

Biol. 162:729-773 (1982), using Sequenase kits (U.S. Biochemical Corp., cleveland, OH). The full-length clones 65 (SEQ. ID. No. 10), 25 (SEQ. ID. No. 4), 7b, 11a (SEQ. ID. No. 2), 29c (SEQ. ID. No. 6) and 6b (SEQ. ID. No. 12) were
5 sequenced completely in both directions with primers spaced approximately 20 bases apart. The remaining positive clones from the high-HLx cDNA library were sequenced in both directions through both the 5' and 3' ends and through all the regions which would identify any of the known allelic
10 variants.

The majority of the clones (50) isolated from the library from liver S33 coded for 2C9. Interestingly, all of the 50 clones appeared to be 1 of 2 2C9 allelic variants, typified by the full-length clones 65 (SEQ. ID. No. 10) and 25
15 (SEQ. ID. No. 4). All of these clones were sequenced through the 5' and 3' ends and through regions which would identify known allelic variants. Thirty-nine of the 2C9 clones were identical with clone 65 (SEQ. ID. No. 10), and 11 were identical with clone 25 (SEQ. ID. No. 10).

20 The nucleotide sequence for clone 65 (SEQ. ID. No. 10) and clone 25 (SEQ. ID. No. 4) is shown in Figure 2. Clones 25 (SEQ. ID. No. 4) and 65 (SEQ. ID. No. 10) were identical in the 5'- and 3'-noncoding regions but contained two single-base changes at positions 1075 and 1425. One of
25 these base changes was conservative, but the second would result in one amino acid difference at position 359 (isoleucine versus leucine). clone 65 (SEQ. ID. N. 9) is identical in amino acid sequence with human form 2, although it differs by two silent changes in the coding region and four
30 differences in the noncoding region (Yasumori et al., 1987). Clone 65 (SEQ. ID. No. 9) contained a leucine instead of a isoleucine at position 4, a valine instead of a serine at position 6, and an arginine instead of a cysteine at position 144 compared to the 2C9 sequenced by Kimura et al. (1987).
35 The 2C9 reported by Meehan et al. has substitutions at positions 144, 175, and 238 compared to the clones obtained in this invention (Meehan et al., *Am J Hum Genet.*, 42:26-37 (1988)).

The remaining clones characterized from the human liver S33 cDNA library encode several novel P450 2C cDNAs. Their DNA sequences are shown in Figure 2 and their percent homology with other known 2C members shown in Table II. Two of these clones, 29c (SEQ. ID. No. 6) and 6b (SEQ. ID. No. 12), differ by one nucleotide in the coding region (position 1154), which would result in a single amino acid change (threonine vs methionine at position 385). Clone 29c (SEQ. ID. No. 6) had a very long (198 bp) 5'-noncoding sequence and a polyadenylation signal 21 bases from the poly (A) tail. Clone 6b (SEQ. ID. No. 12) had an unusually long 3'-noncoding region containing three possible polyadenylation signals with no poly(A) tail. The differences in the 3'-noncoding region could represent alternate splicing, allelic variants, or possibly separate genes. However, these clones are designated as allelic variants of (2C18) because they differ by only one base in the coding region. They are most similar to 2C9 (82% amino acid homology) and 2C19 (SEQ. ID. No. 2) (81% amino acid homology) (Table II).

A third unique P450 2C cDNA, clone 11a (SEQ. ID. No. 2) (designated 2C19), was also identified. 2C19 is 92% homologous in its amino acid sequence to 2C9, 81% homologous to 2C18, and 79% homologous to 2C8 (SEQ. ID. No. 8). Clone 11a (SEQ. ID. No. 2) had a short 5'-leader sequence and contained the stop codon, but did not have a polyadenylation signal or poly(A) tail. Interestingly, no clones for 2C10 (MP-8) were isolated from either library, despite the sequencing of the 3' region of all 50 putative 2C9 clones.

TABLE II

Percent Homology for Nucleotide
and Amino Acid Sequences of P450 2C cDNAs*

Clone	2C8 (SEQ ID NO.8)	2C9	29c (SEQ ID NO.6) (2C18)	11a (SEQ ID NO.2) (2C19)
29c (2C18) (SEQ ID NO.6)	84 89	86 93	100 100	86 93
11a (2C19) SEQ ID NO.2)	83 91	94 96	86 93	100 100

* For each comparison, the upper value represents percent nucleotide homology, and the lower value represents percent amino acid homology. The nucleic acid comparisons include both the coding and 3'-non-coding regions. The 2C9 sequence used in this comparison was the cDNA sequence for clone 65.

Figure 4 shows the alignment comparisons for the deduced amino acid sequences of all known members of the human CYP2C family, including the three new P450s of the present invention. The 7 proteins, along with the consensus sequence, can be aligned with no gaps, and each is predicted to be 490 amino acids long. The amino acid sequences show marked similarities with many regions of absolute conservation. Regions of marked conservation are noted from 131 to 180, and from 302 to 460. These human P450 2C protein sequences also demonstrate hypervariable regions which may be important for interactions between the enzyme and substrate. These include the region from 181-120 and 220-248 as well as 283-296 and a short region near the carboxyl terminus at 461-479. Notably, it has been reported that a putative recognition site for phosphorylation of P450 by cAMP-dependent kinase for P450 2B1 (Arg-Arg-Phe-Ser) at positions 124-127 was conserved in 2C8 (SEQ. ID. No. 8), 2C9, and 11 (2C19), suggesting that these cytochromes might be regulated by phosphorylation (Muller et al., *FEBS Lett.* 187:21-24 (1985)).

However, 2C18 did not contain a serine at this site. The overall percent homology for both nucleic acid and protein sequences is summarized in Table II.

Two additional full-length allelic variants of 2C9 have been isolated. One of these clones is identical with MP-4, but is full-length. It varies from the almost full-length human form 2 isolated by Yasumori et al., *supra*, by only two silent base changes in the coding region and by four changes in the noncoding region. The number of differences in the nucleic acid sequences of the presumed allelic variants isolated by different laboratories range from 4 to 17 and the amino acid changes vary from 0 to 4, as illustrated in Figure 3. Two of the amino acid differences occur within the first six N-terminal residues, the others occurring singly throughout the sequence. The effect of these changes on catalytic activity has not been systematically studied. In Relling et al., *J. Pharmacol. Exp. Ther.* 252:442-447 (1990), it was reported that when the cDNAs for 2C8 (SEQ. ID. No. 8) and 2C9 4-hydroxylated racemic mephenytoin but did not metabolize (S)-mephenytoin. However, the form of isolated 2C9 (human form 2) which is described in Yasumori et al. (1990), metabolized (S)-mephenytoin preferentially when expressed in yeast. These forms differed by only three amino acids. In contrast, Brian et al., *Biochemistry* 28:4993-4999 (1989) found that when a full-length MP-8 (constructed with the first 15 nucleotides predicted from the known amino acid sequence of P450_{mp-1}) was expressed in yeast, it did not metabolize (S)-mephenytoin. This form would differ from human form 2 by only two amino acids. Thus, the role of 2C9 in (S)-mephenytoin metabolism remains controversial.

Example 3: Human RNA Blot Analysis and Hybridization
Conditions

Poly(A+) RNA (10µg) was electrophoresed in a 1% agarose gel under denaturing conditions and transferred to a Nytran filter (Micron Separation, Inc., Westboro, MA), and filters were then baked for 2 h at 80°C. The filters were prehybridized for 2 h, then hybridized overnight with a ³²P-labeled specific oligonucleotide probe for 2C8 (SEQ. ID. No. 8) (T300R) at 42°C, washed 3 x 5 min at room temperature and 1 x 5 min at 42° C with 2 x SSC/0.1% SDS, and

radioautographed. Filters were then stripped with 5 mM Tris (pH 8.0), 0.2 mM EDTA, 0.05% sodium pyrophosphate, and 0.1 x Denhardt's for 2 h at 65° C and rehybridized with a random-primed actin cDNA (Oncor, Gaithersburg, MD) at 50° C using 6 x SSC, 4 x Denhardts, and 0.5% SDS. These filters were washed 1 x 5 min at room temperature, 1 x 10 min at 48° C, and 4 x 15 min at 48° C and radioautographed as before. The 2C8 mRNA band was quantitated by scanning with an LKB Ultrascan laser densitometer, and the values of the integrated peaks were divided by those of the actin peaks.

Hybridization with T300R was negligible in mRNA from 860624 compared to S33 and a number of other liver samples (Figure 5). When corrected for hybridization with the actin probe, the amounts of 2C8 (SEQ. ID. No. 8) mRNA were consistent with the relative amounts of HLx observed in Western blot analysis. Laser scans of the autoradiographs indicated that 2C8 (SEQ. ID. No. 8) mRNA levels in sample 860624 were at least 70-fold lower than in S33 and 3 to 15-fold lower than in any of the remaining samples.

Example 4: Cell Expression Studies

cDNA inserts were ligated into the cloning region of the expression plasmids pSVL (Pharmacia LKB biotechnology, Inc., Piscataway, NJ) or pcD (Okayama et al., *Mol. Cell. Biol.* 3:280-289 (1983)) and used to transform COS-1 cells. COS-1 cells were placed at $(1-2) \times 10^6$ cells per 1-cm dish and grown for 24 h in Dulbecco's-modified Eagle's medium with 10% fetal bovine serum (DMEM). The cells were then washed with Dulbecco's phosphate-buffered saline (PBS) and transfected with recombinant plasmid (3 µg per dish) in DEAE-dextran (500 µg/mL) for 30 min-1 h at 37° C. The transfected cells were then treated with chloroquine (52 µg/mL) in DMEM for 5 h (Luthman et al., *Nucleic Acids Res.* 11:1295-1308 (1983)), washed with PBS, refed with DMEM, and incubated for 72 h prior to harvest. Typically, 15-20 dishes were transfected with each recombinant plasmid. For Western blot analysis of the recombinant transformed COS-1 cells, cells were scraped from the dishes into buffer (50 mM Tris-HCl, pH 7.5, 150mM KCl, and

1mM EDTA) and lysed with 3 x 5 s bursts with a polytron. A portion of each lysate was centrifuged at 9000g and then 10000g for the preparation of a microsomal fraction. Western blots were then performed as described above. Total RNA was isolated from transfected COS-1 cells, and Northern blots were performed as described for human samples. The filters were hybridized with a ³²P-labeled oligonucleotide probe which hybridizes with all 2C clones isolated (2C500R) (5'-GGAGCACAGCCCAGGATGAA-3') (SEQ. ID. No. 20) at 55°C, and radioautographed.

The two variant cDNAs for 2C9, the two variant cDNAs for 2C18, and the cDNA for 2C19 were inserted into expression vectors and transfected into COS-1 cells. Cell lysates were prepared and immunoblotted by using antibody to HLx and P450 2C9. The results are shown in Figure 4. Transfection of COS-1 cells with the two variants of 2C9 (25 (SEQ. ID. No. 4) and 65 (SEQ. ID. No. 10)) resulted in the expression of a protein (SEQ. ID. No. 3) with a molecular weight equal to that of pure 2C9. In contrast, neither 2C18 (either variant) nor 2C19 was detected by antibody to HLx or 2C9. However, Northern blot analysis indicated that all three cDNAs had been successfully transfected into these cells. The sizes of the transcripts were those expected for the constructs. The somewhat lesser hybridization of the 2C oligoprobe with RNA from cells transfected with 11a (SEQ. ID. No. 2) reflects a lower amount of RNA in this sample as shown by the hybridization with the actin probe.

Example 5: Expression of Cytochrome P450 2C19 and 2C18 Polypeptides in a Stable Cell Line

1. Materials

(a) Liver Samples and Chemicals

Human liver samples were obtained from Dr. Fred Guengerich, University of Vanderbilt, Nashville, TN. Restriction endonucleases were purchased from Stratagene Cloning Systems (La Jolla, CA). [α -³²P]dCTP (3000 Ci/mmol), [γ -³²P]ATP (5000 Ci/mmol) and [α -³⁵S]dATP (650 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). Nirvanol was

obtained from Adrian K pfer, University of Berne, Switzerland and separated into its R- and S- enantiomers as described by Sobotka et al., *J. Amer. Chem. Soc.* 54:4697-4702 (1932). Radiolabelled S- and R-mephenytoin (N-methyl-¹⁴C) were
5 synthesized by E.I. DuPont de Nemours & Co., Inc. (Wilmington, DE) by methylation of R- and S-nirvanol. The radiochemical purity of both isomers was greater than 90% as assessed by HPLC. A single impurity which accounted for less than 2% of
10 the parent compound was not characterized, since it eluted after the metabolites and parent compound. Moreover, the percentage of the impurity remained the same (less than 2%) before and after incubations. All sequencing was done by the dideoxymethod using Sequenase Kits (U.S. Biochemical Corp., Cleveland, OH). The specific activities of the S- and R-
15 enantiomers were 20.7 and 20.9 mCi/mmol respectively. All other reagents used are listed below or were of the highest quality available.

20 (b) Additional Sequences of 2C cDNAs Used in the Expression Studies

Two full-length clones of 2C8 (7b and 7c) described in Romkes et al., *Biochemistry* 30:3247-3255 (1991), were sequenced through the coding region in the present study. The sequences were similar to that of the 2C8(HP1-1) reported by
25 Okino et al., *supra*; however, both clones had coding changes at position 390 (A→C) (Asn¹³⁰→Thr) and G→C at position 792 (Met²⁶⁴→Ile) and a change in the noncoding region at 1497(T→C). These changes presumably represent a second allelic variant of 2C8. The Thr¹³⁰ and Ile²⁶⁴ amino acids
30 found in our 2C8 clones are conserved in the remainder of the human P450 2C subfamily (2C9, 2C18, and 2C19) and are therefore consistent with the amino acid substitutions in other members of this subfamily.

35 (c) Yeast Strains and Media

Saccharomyces cerevisiae 334 (MAT α, pep 403, prb1-1122, ura 3-52, leu 2-3, 112, reg1-501,gall), a protease deficient strain kindly provided by Dr. Ed Perkins (NIEHS),

was used as the recipient strain in these studies and propagated non-selectively in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) (Hovland et al., *Gene* 83:57-64 (1989)). For the selection of Leu⁺ transformants, the cells were grown
5 in synthetic complete medium minus leucine (Rose et al., *Methods in Yeast Genetics* (Rose et al., eds.) pp. 180-187, C.S.H.P., NY 1990). Plates were made by the addition of 2% agar.

10 2. Methods

(a) Amplification of 2C18 and 2C9 RNA for Direct Sequencing

Total RNA from selected human liver samples was isolated by the single-step method (Chomczynski et al., *Anal. Biochem.* 163:156-159 (1987), using TRIAGENT™ (Mol. Res. Center, Inc., OH). RNA (10 µg) was reverse transcribed using
15 2.6 µM random hexamers as the 3'-primer by incubating for 1 hour at 42°C using 2.5 U/µl of M-MLV reverse transcriptase (BRL, Grand Island, NY) in 10 mM Tris-HCl, pH 8.3, 5 mM KCl, 5mM MgCl₂, 1 U/µl RNase inhibitor (Promega, Madison, WI) and
20 1 mM each of dATP, dCTP, dGTP, and dTTP (Perkin Elmer Cetus, Norwalk, CT). The samples were then heated for 5 minutes at 99°C to terminate the reverse transcription.

The cDNA was then amplified for a region containing
25 the allelic differences in 2C18 and 2C9 using a nested PCR method. The DNA was amplified in 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.3) containing 1 mM MgCl₂, 0.2 mM each of dATP, dCTP, dGTP, dTTP and 20 pmol of each of the 5' and 3' primers in a final reaction volume of 100 µl. The reaction
30 mixture was heated at 94°C for 5 minutes before addition of 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus). For PCR of 2C18, the 3'-primer was 5'-TGGCCCTGATAAGGGAGAAT-3' (SEQ. ID. No. 23) and the 5'-primers were
5'-ATCCAGAGATACATTGACCTC-3' (SEQ. ID. No. 24) (outer) and
35 5'-CCATGAAGTGACCTGTGATG-3' (SEQ. ID. No. 25) (inner). For 2C9, the 3'-primer was 5'-AAAGATGGATAATGCCCCAG-3' (SEQ. ID. No. 26) and the 5'-primers were 5'-GAAGGAGATCCGGCGTTTCT-3' (SEQ. ID. No. 27) (outer) and 5'-GGCGTTTCTCCCTCATGACG-

3'(SEQ. ID. No. 28) (inner). The outer amplification was performed for 20 cycles consisting of denaturation at 94°C for 1 minute, annealing at the appropriate temperature for 30 seconds, and extension at 72°C for 1 min. After a 50-fold dilution, PCR was carried out similarly with the inner primers for 35 additional cycles.

The PCR products were purified using a Centricon-30, dried, suspended in 40 µl of sterile water, and sequenced using Sequenase Kits and a P³³-end labeled sequencing primer. For 2C18, the primer used was 2C18.1184R 5'-TTGTCATTGTGCAG-3' (SEQ. ID. No. 29). Sequencing primers for 2C9 were 2C9.1030F 5'-CACATGCCCTACACA-3' (SEQ. ID. No. 30), 2C9.385F 5'-TGACGCTGCGGAATT-3' (SEQ. ID. No. 31), and 2C9.783F 5'-GGACTTTATTGATTG-3 (SEQ. ID. No. 32).

Full length 2C9 cDNA was also amplified by PCR from a human liver with high S-mephenytoin 4'-hydroxylase activity using the primers 5'-ATGATTCTCTGTGGTCCT-3' (SEQ. ID. No. 33) and 5'-AAAGATGGATAATGCCCCCAG-3' (SEQ. ID. No. 34). The PCR reaction was similar to above, except that the primer concentrations were increased 10-fold (0.25 µM). The PCR products were then cloned into the pCR1000 vector using the TA Cloning System (In Vitrogen, San Diego, CA) and sequenced to identify the allelic variant present.

(b) Plasmid Construction and Methods for Amplifying Full-length 2C18 and 2C19 cDNAs by PCR

The strategy for cloning the P450 2C cDNAs into the yeast vector pAAH5 is described below. The 5'-noncoding sequence of the P450 2C cDNAs was eliminated by PCR amplification to optimize expression in yeast cells. The 5'-primer introduced a Hind III cloning site and a six A-residue consensus sequence upstream of the ATG codon to promote efficient translation in yeast (Hamilton et al., Nucl. Acids Res. 15:3581-3593 (1987), Cullin et al., Gene 65:203-217 (1988)). The 3'- primer was positioned between the stop codon and polyadenylation site and introduced a second Hind III site. cDNA inserts in the pBluescript vector (0.1 µg) (Romkes et al., (1991), supra) were amplified by PCR as described

before except that the reaction contained 3.5 mM MgCl₂, 0.25 μM each of the 5'- and 3'- primers, and 1 μl PerfectMatch (Stratagene, La Jolla, CA). Amplification was performed in sequential cycles, with the first cycle including denaturation for 1 min. at 94°C, annealing at the appropriate temperature for 1 min., and polymerization at 72°C for 3 min. The remaining 24 cycles consisted of a denaturation step at 94°C for 1 min. and a combined annealing/extension step at 72°C for 3 min. After the last cycle, all samples were incubated an additional 10 min. at 72°C. The primers used were:

2C8: 5'-GCAAGCTTAAAAAATGGAACCTTTTGTGGTCCT-3' (SEQ. ID. No. 35) and 5'-GCAAGCTTGCCAGATGGGCTAGCATTCT-3' (SEQ. ID. No. 36); 2C9: 5'-GCAAGCTTAAAAAATGGATTCTCTTGTGGTCCT-3' (SEQ. ID. No. 37) and 5'-GCAAGCTTGCCAGGCCATCTGCTCTTCT-3' (SEQ. ID. No. 38); 2C19: 5'-GCAAGCTTAAAAAATGGATTCTCTTGTGGTCCT-3' (SEQ. ID. No. 39) and 5'-GCAAGCTTGCCAGACCATCTGTGCTTCT-3' (SEQ. ID. No. 40).

The PCR products were cloned into the pCR1000 vector (Invitrogen, San Diego, CA). Recombinant plasmids were isolated from *E. coli* (INVαF') cells using Qiagen plasmid purification kits, and the PCR products were completely sequenced as described above to verify the fidelity of the PCR reaction. A mutation of ASP²→Val was initially introduced inadvertently in 29c via the primers utilized due to an error in the original sequencing at this position. Therefore, the correct 2C18-Asp² cDNAs were cloned into the pAAH5 vector by an alternate strategy. The 3'-end was cut with NdeI, blunted, and ligated to a SmaI/HindIII adapter. The clone was then partially digested with BamHI which cuts after the initiation ATG as well as internally, and the intact 1700 fragment get purified. A BamHI/HindIII linker was prepared from the oligos 5'-AGCTTAAAAAATG-3' (SEQ. ID. No. 41) (upper) and 5'-GATCCATTTTTTTA-3' (SEQ. ID. No. 42) (lower), annealed, and ligated to the cDNA fragment to introduce a HindIII cloning site and regenerate the ATG codon.

The PCR amplified cDNAs were isolated by Hind III digestion, ligated into the pAAH5 yeast expression vector, and the proper orientation confirmed by restriction analysis and

sequencing. The expression vector pAAH5, which contains the yeast ADH1 promoter and terminator regions and the Leu2 selectable marker, was kindly provided by Dr. M. Negishi (NIEHS). The recombinant plasmids were isolated from *E. coli* Dh5 α cells using Qiagen plasmid purifications kits and transformed into yeast as described previously (Faletto et al., *J. Biol. Chem.* 267:2032-2037 (1992), using the lithium acetate method of Ito et al., *J. Bacteriol.* 153:163-168 (1983).

(c) Immunoblots and Cytochrome P450 Determinations

Yeast microsomes or whole cell lysates were prepared from transformed cells isolated at mid-logarithmic phase as described previously (Oeda et al., *supra*) with slight modifications (Faletto et al., *supra*) and stored at -80°C in 0.1 M phosphate (pH 7.4) containing 20% glycerol and 0.1 mM EDTA. Protein concentrations were determined by the method of Bradford et al., *Anal. Biochem.* 72:248-254 (1976). SDS-polyacrylamide gel electrophoresis and Western blots were performed on yeast microsomes or whole cell lysates (Faletto et al., *supra*) and immunoblots probed with antibody to the appropriate P450 as described (Yeowell et al., *Arch. Biochem. Biophys.* 243:408-419 (1985). Cytochromes P450 2C8, P450 2C9 and NADPH:P450 reductase were purified from human liver microsomes (Raucy et al., *Methods in Enzymol.* 208:577-587 (1991) and antibodies to 2C8 and 2C9 prepared in rabbits as previously described (Leo et al., *Arch. Biochem. Biophys.* 269:305-312 (1988)). Specific peptides NH₂-CIDYLPGSHNKIAENFA-COOH (SEQ. ID. No. 43) (amino acids 231-249) for P450 2C18 and NH₂-CLAFMESDILEKVK-COOH (SEQ. ID. No. 44) (amino acids 236-249) for 2C19 were selected from amino regions where these P450s vary from other known 2C subfamily members (Romkes et al., (1991), *supra*). These peptides were synthesized, conjugated to bovine serum albumin via *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, and antibodies to the conjugates raised in rabbits by BIOSYNTHESIS INC. (Denton, TX). *E. coli* lysate (4 mg/ml) was added to the primary peptide antibody in first step of the immunoblot procedure to block non-specific

reactions of these rabbit antibodies to yeast cell wall proteins. Cytochrome P450 concentrations of microsomes were determined by dithionite-reduced carbon monoxide difference spectra by the method of Omura et al., *J. Biol. Chem.* 239:2370-2378 (1964) using an extinction coefficient of 91 mM⁻¹cm⁻¹.

Microsomes of human livers were prepared as described by Raucy et al., *supra*. SDS-polyacrylamide gel electrophoresis and immunoblot analysis was performed as above except that immunoblots were developed using the ECL (enhanced chemiluminescence) Western blotting kit from Amersham (UK). Immunoblots were scanned with a laser densitometer (LKB Instruments).

15 (d) Purification of Cytochromes from Recombinant Yeast Microsomes

Recombinant yeast microsomes were prepared from a 10-12 l culture, and recombinant P450s were purified by aminooctylsepharose chromatography as described by Iwasaki et al., *J. Biol. Chem.* 226:3380-3382 (1991). The Emulgen was then removed from protein by adsorption of the protein to a 4g hydroxylapatite column (Hypatite C, Clarkson Chemical Company, Williamsport, PA) equilibrated with 10 mM potassium phosphate buffer (pH 7.2), 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT and washing the column with the same buffer until the absorbance at 280 nm returned to zero. The P450 was then eluted with 4090 mM DTT, and dialyzed overnight against 100 mM potassium phosphate buffer (pH 7.4, 20% glycerol and 0.1 mM EDTA. Absolute and CO difference spectra of purified P450s were determined in the same buffer but containing 0.2% Emulgen and 0.5% cholate.

(e) Tolbutamide Hydroxylase Assays

Tolbutamide hydroxylase activity was measured according to Knodell et al., *J. Pharmacol. Exper. Ther.* 241:1112-1119 (1987), with several modifications. Yeast microsomes (1 mg protein) were preincubated with 300 pmol hamster P450 reductase in 0.2 ml of the incubation buffer

(below) for 3 min at 37°C. The reaction was then placed on ice and incubated in 0.2 ml of 50 mM HEPES buffer (pH 7.4) containing 1.5 mM MgCl_2 , 0.1 mM EDTA in a final volume of 1 ml and 1 mM sodium tolbutamide. The reaction was initiated with 0.5 mM NADPH. Human liver microsomes (0.22 mg protein) were incubated without reductase. Incubations with reconstituted recombinant P450s contained 50 pmol purified P450 enzyme, 150 pmol P450 reductase, and 15 μg dilauroylphosphatidylcholine, and were performed in 100 mM potassium phosphate buffer (pH 7.4). Reactions were terminated after 60 min at 37°C by the addition of 50 μl of 4N HCl, followed by extraction with 3 ml of water-saturated ethyl acetate. The ethyl acetate extracts were dried under nitrogen at 40°C, the residue resolubilized in 200 μl methanol, and 4-hydroxytolbutamide then assayed using HPLC by injecting 50 μl of the solubilized extract onto a $\mu\text{BONDAPAK C}_{18}$ column (4.6x300 mm) using 0.05% phosphoric acid, pH 2.6: acetonitrile (6:4, v/v) as the mobile phase with a flow rate of 1 ml/min. The column eluate was monitored at 230 nm and rates of product formation were determined from standard curves prepared by adding varying amounts of 4-hydroxytolbutamide to incubations conducted without NADPH. Preliminary experiments confirmed that 4-hydroxytolbutamide formation by human liver microsomes (30-120 pmol P450) was linear for up to 90 min. Samples were analyzed in triplicate.

(f) Mephenytoin 4'-Hydroxylase Assay

Mephenytoin 4'-hydroxylase activity was measured by a modification of the radiometric HPLC assay described by Shimada et al., *J. Biol. Chem.* 261:909-921 (1986), as described below. Purified or recombinant yeast microsomes (10-50 pmol) were preincubated with dilauroylphosphatidylcholine (15 μg per 50 pmol P450), P450 reductase (500 U per 50 mol P450), and human cytochrome b_5 (2:1 molar ratio when added). The reconstituted mixture was preincubated for 5 min at 37°C, and then placed on ice. A final concentration of 0.4 mM radiolabelled S- or R-mephenytoin (20.7 mCi/mM and 20.9 mCi/mMol) was added to 50 mM

HEPES buffer (pH 7.4) containing 0.1 mM EDTA and 1.5 mM MgCl_2 for recombinant 2C proteins. The mixture was then incubated at 37° with shaking for 3 min, and the reaction started with the addition of 2mM NADPH and terminated after 30 min with an equal volume of methanol. Cytochrome b_5 was not included in all CYP2C18 reactions, since it had no effect or produced a slight inhibition on the activity of this CYP protein. Reaction volumes were generally 0.25 ml except when the volume of recombinant purified cytochrome or yeast microsomes was greater than 50 μl . In these cases, the volume was increased to 0.5 ml to limit the volume of glycerol from the purified preparation to <4% of the final volume. Incubations with human microsomes did not contain exogenous P450 reductase or cytochrome b_5 , and they were carried out in 0.1 M phosphate buffer (pH 7.4) instead of HEPES buffer. Initial experiments shows that S-mephenytoin hydroxylase activity of human liver microsomes was linear for at least 60 minutes and from 0.05 through 0.2 mg microsomal protein, and that of the R-enantiomer was linear through 1 mg microsomal protein.

At the end of the incubation period, the reactions were terminated with an equal volume of methanol. The incubation mixture was centrifuged at 10,000g for 10 min and an aliquot assayed directly using HPLC without extraction. Samples with particularly low activity were concentrated by lyophilization and redissolved in a small volume of methanol:water (1:1) before assay. The HPLC system consisted of a reverse phase C18 (10 μm) Versapak, 300 mm x 4.1 mm column (Altech Associates, Deerfield, IL) using an isocratic solvent consisting of methanol:water (45:55) with a flow rate was kept of 1 ml/min for 25 min. Detection of radioactive peaks was accomplished using an on-line Flow-One radiochemical detector (Radiomatic Instruments Co., Tampa, FL. Detection of the unlabeled 4'-hydroxymephenytoin authentic standard was performed using an on-line multiwavelength UV detector at both 211 and 230 nm.

(g) Statistical analyses

Tolbutamide hydroxylase and mephenytoin hydroxylase activities of microsomes prepared from different recombinant yeasts were compared by analysis of variance and by Fisher's least significant difference test (Carmer et al., Am. Stat. Ass. 68:66-74 (1973)).

3. Results(a) Expression of P450 2C cDNAs in yeast

Western blot analysis confirmed the expression of the recombinant human CYP2C proteins in the recombinant yeast (Fig. 6). Antibodies to 2C8 and 2C9 recognized polypeptide bands of approximately 50,000 daltons (2C8) and 55,000 daltons (2C9) which corresponded in mobility to those of the recombinant proteins purified from yeast microsomes. These mobilities corresponded to those of the corresponding 2C8 and 2C9 proteins purified from human liver. 2C19 was recognized by antibodies to both the 2C9 and the 2C19 peptides. This protein corresponded in mobility (<50,000 daltons) to the lowest of three bands in Western blots of human liver microsomes probed with antibody to human 2C9. The mobility of 2C18 was intermediate between that of 2C8 and 2C19. Antibodies to 2C18 and 2C19 peptides were specific for their antigen; however, antibody to 2C9 cross-reacted strongly with 2C19 and weakly with 2C8 and 2C18.

CO difference spectral analysis indicated that the recombinant P450 2C proteins were expressed at levels as high as 160-250 pmol/mg protein in some yeast microsomal preparations. 2C18, 65 (2C9), and 25 (2C9) were expressed at levels of 20 to 60 pmol/mg microsomal protein. Initially, 11a (2C19) was expressed extremely poorly, and the CO difference spectrum of the recombinant 2C19 yeast was indistinguishable from that of control yeast (<7 pmol/mg protein). However, after repeated transfections and selection, expression of 2C19 at 17 pmol/mg protein was achieved. All of the CYP2C proteins were low spin hemoproteins. CYP2C18 appeared to be somewhat unstable in yeast microsomes with a large proportion (~1/3 to 1/2) of the P450 being converted to P420 in the

presence of dithionite and carbon monoxide. None of the other recombinant CYP2C proteins showed this lack of stability.

(b) Optimization of Tolbutamide and S-Mephenytoin

5 Hydroxylase Assays

Preliminary studies indicated that exogenous P450 reductase (500 U/50 pmol P450) stimulated metabolism of tolbutamide by recombinant 2C9 in yeast microsomes > 10-fold and stimulated S-mephenytoin hydroxylase activity
10 approximately 2-fold. Activity of the recombinant 2C proteins was linear with amount of P450 for 30 minutes through at least 20 pmol P450 for 2C19 (Fig. 7) and 50 pmol for the other CYP2C forms. Cytochrome b₅ stimulated S-mephenytoin hydroxylase activity of both 2C9 and 2C19 in yeast microsomes and the
15 optimal ratio of b₅ to P450 was approximately 2:1, but it generally had no effect or produced a slight inhibition of mephenytoin hydroxylase activity of 2C18 (Fig. 8). This difference is consistent with the fact that all of the CYP2C proteins except 2C18 contain a Ser at position 128 which is a
20 recognition site for cAMP protein kinase (¹²⁵Arg-Arg-Phe-Ser¹²⁸) (Müller et al., *FEBS Lett.* 187:21-24 (1985), and this sequence is also thought to be part of a b₅ binding site (Jansson et al., *Arch. Biochem. Biophys.* 259:441-448 (1987); 2C18 contains Cys at position 125.

25 Mephenytoin 4'-hydroxylase activity of recombinant yeast microsomes was consistently higher in HEPES than phosphate buffer, while activity of human liver microsomes was ~2-fold higher in phosphate buffer (pH 7.4). Therefore, recombinant proteins were subsequently assayed in HEPES buffer
30 with exogenous reductase and cytochrome b₅ except for 2C18 which was tested both with and without cytochrome b₅. Human liver microsomal activities were assayed in phosphate buffer.

(c) Mephenytoin hydroxylase activity of recombinant human 2C proteins

S-mephenytoin 4'-hydroxylase activities of yeast microsomes containing recombinant human CYP2C proteins were compared under optimized conditions described above. HPCL profiles of the metabolites of S-mephenytoin produced by human liver microsomes and recombinant human CYP2C proteins are shown in Fig. 9 and the results summarized in Table III. Recombinant 2C19 4'-hydroxylated S-mephenytoin at a rate of ~5 nmol/min/nmol P450 which was an order of one magnitude higher than the rate of 4'-hydroxylation in human liver microsomes (Table III and Fig. 9). The retention time (5-6 min) of the 4'-hydroxymephenytoin metabolite was identical to that of the authentic unlabeled standard. 2C19 also produced small quantities of two unknown metabolites eluted at 3-4 and 7-8 min. These unknown metabolites were also produced by liver microsomes, and the metabolite with the shorter retention time was the principal metabolite produced by 2C8. Parent S-mephenytoin eluted at 14-15 min. followed by the unknown impurity which eluted at 16-17 min. Similar retention times were observed for R-mephenytoin and its metabolites.

The rate of 4'-hydroxymephenytoin formation by 2C19 was at least 100-fold higher than that of 2C9 (both alleles), 2C18 (both alleles) and 2C8 (Table III). The rate of 4'-hydroxylation of S-mephenytoin by 2C8 appeared to be lower than that of 2C9 (0.02 nmol/min/nmol). The 4'-hydroxylation of mephenytoin by 2C19 was stereospecific; the rate of S-hydroxylation was at least 30-fold higher than that of R-hydroxylation (Table III). In contrast, the 4'-hydroxylation of mephenytoin by the other human CYP2C proteins did not appear to be stereospecific.

TABLE III

S-Mephenytoin 4'-Hydroxylase Activities in
Recombinant Human CYP2C Yeast Microsomes

Microsomes	Mephenytoin 4'-Hydroxylase Activity nmol/min/nmol P450			R/S Ratio
	S	R		
Controls	0.028 ± 0.001	0.024 ± 0.003		0.9
2C9-Ile ³⁵⁹ (65)	0.043 ± 0.000	0.041 ± 0.005		0.9
2C9-Leu ³⁵⁹ (25)	0.031 ± 0.009	0.040 ± 0.01		1.3
2C8	0.037 ± 0.001	0.016 ± 0.001		0.4
2C18-Thr ³⁸⁵ (29c) + b5	0.042 ± 0.004	0.054 ± 0.003 ^a		1.3
2C18-Thr ³⁸⁵ (29c), no b5	0.034 ± 0.008	-		
2C18-Met ³⁸⁵ (6b)	0.023 ± 0.004	0.019 ± 0.005		0.9
2C19 (11a)	4.6 ± 0.3 ^{a,b,d}	0.014 ± 0.02 ^a		0.03
Human liver microsomes HB1 6	0.283 ± 0.037 ^{a,c,d}	0.117 ± 0.017 ^{a,c}		0.4

S-Mephenytoin hydroxylase assayed as described in Methods. Reaction mixtures contained 10 pmol of recombinant CYP2C19 or 50 pmol of other recombinant CYP2C yeast microsomes, 500 U of purified P450 reductase and 15 µg phospholipid per 50 pmol of P450, and 0.4 mM radioactive substrate in 0.1 M HEPES buffer (pH 7.4). Unless otherwise stated recombinant yeast microsomes were also reconstituted with a 2:1 molar ratio of cytochrome b₅. Reactions were incubated at 37°C for 30 min with 1 mM NADPH. Control reactions contained the same reaction mixture and were incubated similarly with an equivalent amount of control yeast microsomal protein (1 mg). Specific content of P450 of the recombinant yeast microsomes ranged from 35-48 pmol/mg except for 2C8 (191 pmol/mg) and 2C19 (17 pmol/mg). Control liver reactions contained 0.1 mg microsomal protein but were not fortified with reductase, cytochrome b₅, or phospholipid and were incubated with 0.1 M phosphate buffer (pH 7.4). Values represent the means ± SE.

^a Activity significantly higher than that of control yeast microsomes, P < 0.05. Analysis of variance and Fisher's Least Significant difference test.

^b 2C19 activity significantly higher than activities of all other recombinant CYP2C proteins or human liver microsomes, P < 0.05.

^c Human liver microsomes significantly higher than recombinant microsomes except 2C19, P < 0.05.

^d Significant difference between S- and R-Mephenytoin hydroxylase activities, P < 0.05.

TABLE IV
Mephenytoin 4'-Hydroxylase and Tolbutamide Hydroxylase Activities
of Purified Recombinant Human P450s from the 2C Subfamily

P450 2C (clone)	Mephenytoin 4'-Hydroxylase Activity		Tolbutamide Hydroxylase Activity	
	S	R	R/S Ratio	(pmol/min/nmol P450)
2C19	6.17 ± 0.24 ^{a,b,c}	0.19 ± 0.04 ^a	0.03	ND
2C9-Ile ³⁵⁹ (65)	0.081 ± 0.006 ^a	0.063 ± 0.003 ^a	0.77	122 ± 29 ^{a,d}
2C9-Leu ³⁵⁹ (25)	ND	ND		10 ± 2
2C18-Asp ² Thr ³⁸⁵ (29c-1a)	0.116 ± 0.010 ^a	0.147 ± 0.025 ^a	1.3	ND
2C18-Val ² Thr ³⁸⁵ (29c)	0.019 ± 0.001	0.073 ± 0.009 ^{a,c}	2.7	102 ± 2 ^{a,d}
2C18-Asp ² Met ³⁸⁵ (6b)	0.103 ± 0.016 ^a	0.107 ± 0.005 ^c	1.0	ND
2C8	0.057 ± 0.009 ^{a,c}	0.023 ± 0.004	0.4	12 ± 4
2C8 Purified from Human Liver	0.032 ± 0.003	0.051 ± 0.030	1.6	ND
2C9 Purified from Human Liver	0.033 ± 0.001	0.051 ± 0.007 ^{a,c}	1.6	109 ± 16 (390, 2,840) ^{a,d,c}
Human Liver Microsomes HB16	0.46 ± 0.02 ^a	0.28 ± 0.01	0.6	ND
Human Liver Microsomes UC8936	-	-		408 ± 21

Recombinant P450s were purified from yeast microsomes and assays performed as described in Methods. 2C9 were purified from human liver (Raucy and Lasker, 1991). Assays were performed in triplicate and values represent means ± SE. Blank reactions (containing all components except the P450) were subtracted (22 ± 5) from tolbutamide hydroxylase values. Blank reactions for the S-mephenytoin assay were not subtracted since no distinct peaks with the exact retention times of 4'-hydroxymephenytoin were observed; however, background radioactivity was in the range of ~0.025 ± 0.01 nmol/min/nmol. ND=Not determined.

^a Increased over blank values, P < 0.05

^b S-Mephenytoin hydroxylase activity of 2C19 significantly greater than all other values, P < 0.05

^c S-Mephenytoin hydroxylase activity of significantly different from R-mephenytoin values, P < 0.05.

^d Tolbutamide hydroxylase activity of 65 and 29c were significantly greater than 25, or 2C8 (P < 0.0001).

^e Tolbutamide hydroxylase activity of two other 2C9 preparations derived from different human livers in parentheses.

Recombinant CYP2C proteins were purified from yeast microsomes and their ability to 4'-hydroxylate the S- and R-enantiomers of mephenytoin were also examined in a reconstituted system (Table IV). 2C19 had similar turnover numbers for S-mephenytoin 4'-hydroxylation in the reconstituted system and in recombinant yeast microsomes fortified with reductase. This turnover number was at least 10-times higher than that of human liver microsomes, and it was 50-100 times higher than that of recombinant 2C9, 2C18 or 2C8. The turnover number of recombinant 2C9 was ~100 times higher than the activity of a preparation of 2C9 purified from human liver. 4'-hydroxylation of mephenytoin by 2C19 was stereospecific for the S-enantiomer, while metabolism by 2C9 was not stereospecific. Surprisingly, 2C18 appeared to be stereoselective for the R-enantiomer of mephenytoin. The turnover number of 2C19 for S-mephenytoin 4'-hydroxylase was also ~30 times higher than the turnover numbers reported for a preparation P450_{MP} purified from human liver by Srivastava et al., *Mol. Pharmacol.* 40:69-79 (1991) (0.21 nmol/min/nmol P450).

Although 2C9 exhibits poor catalytic activity toward S-mephenytoin, this cytochrome appears to be the principal tolbutamide hydroxylase (Table IV and V). The turnover numbers for hydroxylation of tolbutamide by the purified recombinant 2C9 were somewhat lower than those of 2C9 purified from human liver in the absence of exogenous reductase. The Ile³⁵⁹ allele of 2C9 had a 3-fold higher turnover number for tolbutamide than the Leu³⁵⁹ allele when activity of the recombinant microsomes were adjusted for P450 content (Table V). 2C19 also appeared to metabolize tolbutamide at a rate comparable to that of 2C9, although this rate was difficult to estimate due to the low specific content of P450 in the recombinant 2C19 yeast clone available at the time of these assays. The two alleles of 2C18 exhibited lower tolbutamide hydroxylase activity than 2C9 in recombinant yeast microsomes.

TABLE V

Tolbutamide Hydroxylase Activities of
Recombinant Human CYP2C Yeast Microsomes

5	Microsomes	P450 Content (pmol/mg)	Tolbutamide Hydroxylase Activity	
			(nmol/min/mg protein)	(nmol/min/nmol P450)
	Control Yeast	<5	0.3 ± 0.01	-
	2C9-Ile ³⁵⁹ (65)	55	169.8 ± 7.4 ^{a,b}	3.4 ± 0.15
10	2C9-Leu ³⁵⁹ (25)	20	14.8 ± 0.3 ^{a,c}	0.99 ± 0.02
	2C8	80	8.5 ± 0.2 ^a	0.11 ± 0.003
	2C18-Asp ² Thr ³⁸⁵ (29c-1a)	53	9.3 ± 0.7 ^a	0.19 ± 0.02
	2C18-Asp ² Met ³⁸⁵ (6b-9)	34	11.1 ± 1.2 ^a	0.37 ± 0.04
	2C19 (11a-3)	<7	18.4 ± 2.4 ^{a,d}	ND
15	UC8936 Human Liver Microsomes	227	116 ± 0.8 ^a	2.3 ± 0.02

20 Tolbutamide hydroxylase activities measured as described in methods. Reaction mixtures contained 1 mg yeast microsomal protein or 0.2 mg UC8936 human liver microsomal protein (50 pmol P450). Purified P450 reductase (1,000 units) was included in reactions with yeast microsomes but not human microsomes. Values were the means ± SE. ND=Not calculated due to low specific content of 2C19 in yeast in this experiment.

25 ^a Significantly higher than control yeast microsomes, P<0.05. Pairwise comparisons using Fisher's Least Significant Difference test.

^b Clone 65 significantly higher than all other clones (P<0.0001).

^c Clone 25 significantly greater than 2C8 (P<0.0005).

^d Clone 11a significantly higher than 2C8 (P<0.0001).

30 The data show that CYP2C19 stereospecifically
hydroxylates S-mephenytoin at the 4'- position at a rate which
is at least 10 times higher than the rate in human liver
microsomes. This is the first example of a human CYP protein
which metabolizes S-mephenytoin with a turnover number
35 appreciably higher than that of human liver microsomes. Other
2C proteins showed a 100-fold reduced activity relative to
2C19. One of the 2C9 variants tested (Ile³⁵⁹) is identical to
that reported by Yasumori et al., supra to show a low level of
S-mephenytoin 4'-hydroxylase activity. The low rate of 4'-
40 hydroxylation of S-mephenytoin by 2C9 detected in the present

study with high specific activity ^{14}C -labeled S-mephenytoin undoubtedly explains the conflicting reports from various laboratories concerning the ability of this cytochrome to metabolize mephenytoin (Yasumori et al., *supra*; Srivastava et al., *supra*; Relling et al., *supra*).

(d) Comparisons of Immunoblot Analysis of CYP2C Proteins in Human Livers with Liver Microsomal S-Mephenytoin 4'-Hydroxylase Activities

Microsomes from 16 human liver donor samples previously assayed for S- and R-mephenytoin 4'-hydroxylase activities were analyzed for CYP2C proteins by Western blot analysis (Fig. 10) using an antibody to 2C8 and a polyclonal antibody to 2C9 and 2C19. Both 2C18 and 2C19 have mobilities similar to that of the low molecular weight band recognized in human microsomes by most antibodies to 2C9. However, an antibody to a 2C19 peptide was specific for 2C19. 2C18 could not be detected in human liver samples using a peptide antibody to 2C18 (~5 pmol detection limit), indicating that this polypeptide is expressed poorly (<50 pmol/mg).

The 2C19 content of liver microsomes was consistent with their S-mephenytoin 4'-hydroxylase activities (Fig. 10). In particular, samples 129 and 130 had extremely low S-mephenytoin 4'-hydroxylase values, low S/R ratios, and 2C19 appeared to be essentially absent in these microsomal samples. Densitometric analysis of immunoblots revealed that 2C19 content of the 16 human liver microsomes correlated significantly with S-mephenytoin 4'-hydroxylase activity ($r=0.718$, $P<0.005$) (Fig. 11), but that the content of 2C9 did not correlate with this catalytic activity ($r=0.49$, $P>0.05$). There was also a significant correlation between 2C8 content and S-mephenytoin 4'-hydroxylase activity ($r=0.82$, $P<0.0001$). However, this correlation was probably fortuitous, because 2C8 shows very low S-mephenytoin 4'-hydroxylase activity either in recombinant form or when purified from human liver. Alternatively, the correlation may indicate an indirect regulatory role for 2C8 in controlling S-mephenytoin 4'-hydroxylase activity.

(e) Sequences of 2C9 and 2C18 mRNAs in Livers with High or Low S-mephenytoin 4'-Hydroxylase Activities

2C18 and 2C9 mRNAs from six of the above livers were amplified by PCR and directly sequenced through areas of known allelic variation to determine whether there was a relationship between S-mephenytoin 4'-hydroxylase activity and the presence of a particular allelic variant (Table VI). When the total 2C18 PCR products were sequenced, the two individuals with the highest S-mephenytoin hydroxylase activity were homozygous for Thr³⁸⁵(ACG). Of the two individuals with the lowest activity, one was homozygous for Met³⁸⁵, and one was heterozygous for Thr/Met³⁸⁵(AC/TG). Two individuals with intermediate activity were also homozygous for Thr³⁸⁵. Similarly, when 2C9 mRNA from these same individuals was amplified and sequenced through known allelic variations, sample 108 (low S-mephenytoin 4'-hydroxylase activity) was heterozygous at C/T⁴³⁰ (coding for Cys/Arg¹⁴⁴), while the other five individuals were homozygous for C⁴³⁰ (Arg¹⁴⁴). Sequencing samples through bases 1072-1077, all samples except for 106 (high activity) read ¹⁰⁷²TACATT¹⁰⁷⁷, coding for Tyr³⁵⁸Ile³⁵⁹. Sample 106 read TACA/CTT indicating that it was heterozygous for Ile/Leu³⁵⁹. These data indicate that there is no relationship between S-mephenytoin 4'-hydroxylase activity of human liver microsomes and the identity of the allelic variants of 2C18 (Thr/Met³⁸⁵) or 2C9 (Arg/Cys¹⁴⁴, Tyr/Cys³⁵⁸, Ile/Leu³⁵⁹) in these tissues.

TABLE VI

Alleles in Human Livers with Varying S-Mephenytoin
4'-Hydroxylase Phenotypes

5	Pheno- type	S-MPOHase nmol/ min/mg	Liver donor	2C18 allele	2C9 allele			
	High	0.286	106	Thr ³⁸⁵	Arg ¹⁴⁴	His ²⁷⁶	Tyr ³⁵⁸	Ile/Leu ³⁵⁹
	High	0.351	115	Thr ³⁸⁵	Arg ¹⁴⁴	His ²⁷⁶	Tyr ³⁵⁸	Ile ³⁵⁹
10	Inter- mediate	0.070	118	Thr ³⁸⁵	Arg ¹⁴⁴	His ²⁷⁶	Tyr ³⁵⁸	Leu ³⁵⁹
	Inter- mediate	0.081	123	Thr ³⁸⁵	Arg ¹⁴⁴	His ²⁷⁶	Tyr ³⁵⁸	Ile ³⁵⁹
	Low	0.051	108	Thr/Met ³⁸⁵	Arg/Cys ¹⁴⁴	His ²⁷⁶	Tyr ³⁵⁸	Ile ³⁵⁹
15	Low	0.025	129	Met/Met ³⁸⁵	Arg ¹⁴⁴	His ²⁷⁶	Tyr ³⁵⁸	Ile ³⁵⁹

4. Conclusion

These results show that 2C19 has a turnover number for the 4'-hydroxylation of S-mephenytoin about 100-fold higher than that of 2C9, 2C18, or 2C8. 2C19 hydroxylation was stereospecific for the S- enantiomer. The hepatic content of 2C19 in 16 liver microsomal samples correlated with their S-mephenytoin 4'-hydroxylase activities. 2C9 appeared to be the primary tolbutamide hydroxylase, although 2C19 may also contribute to this catalytic activity. The identity of the allelic variant of 2C9 or 2C18 did not influence S-mephenytoin 4'-hydroxylase activity. These data strongly indicate that 2C19 is the key determinant of S-mephenytoin 4'-hydroxylase activity in human liver.

30 Example 6: Diagnostic Assays for Detecting Individuals Deficient in S-Mephenytoin 4'-Hydroxylase Activity

Individuals deficient in S-mephenytoin 4'-hydroxylase activity are identified by comparing analysis of their genomic or cDNA encoding 2C19.

(a) Analysis of full-length cDNA

Liver microsomes were prepared by standard differential centrifugation methods (2) from human liver samples previously characterized as varying markedly in S-mephenytoin 4'-hydroxylase *in vitro*. Total liver RNA was isolated from the liver samples with trireagent (Molecular Research Center, Inc.) and reversed transcribed using random hexamers as 3' primers. Overlapping CYP2C19 cDNA fragments from five human liver samples that showed poor metabolism of S-mephenytoin *in vitro* were amplified by the polymerase chain reaction (PCR). PCR was performed on an aliquot of the cDNA in 1 X PCR buffer (67 mM Tris-HCl pH 8.8, 17 mM (NH₄)₂SO₄, 10 mM β -mercaptoethanol, 7 μ M EDTA, 0.2 mg bovine serum albumin/ml), 50 μ M dATP, dCTP, dGTP and dTTP, 0.25 μ M of both PCR primers, 2.5 U AmpliTaq DNA polymerase (Perkin Elmer Cetus) and 1.0 mM MgCl₂. The PCR conditions were: initial denaturation at 94°C for 3 min; 35 cycles consisting of: denaturation at 94°C for 30 sec, annealing at 53°C for 30 sec and extension at 72°C for 30 sec; final extension at 72°C for 10 min; using a Perkin Elmer thermocycler. PCR products (20 μ l) were analyzed on 3% agarose gels stained with ethidium bromide.

The PCR fragments were purified using Microcon filters (Amicon Inc.) and used in the cycle sequencing reaction employing fluorescence-tagged dye terminators (PRISM, Applied Biosystems) and sequenced. One partial CYP2C19 cDNA was isolated which exhibited aberrant splicing of exon 5 (Fig. 12). This cDNA was missing the initial 40 bases of exon 5, and was also missing a SmaI site (Fig. 12). This deletion would be predicted to produce an early stop codon resulting in a truncated defective protein.

(b) Rapid Assay for Identifying 40 bp Deletion in cDNA

The analysis of full-length cDNAs identified a 40 bp deletion as a likely cause of S-mephenytoin 4'-hydroxylase activity deficiency. A rapid assay was therefore devised to

analyze the specific region of a 2C19 cDNA molecule spanning the 40 bp deletion.

Specific PCR primers were designed to amplify the region of the CYP2C19 cDNA spanning the deletion (Figs. 12 and 13). mRNA from 13 human livers previously characterized for extensive or poor metabolism of S-mephenytoin *in vitro* were reverse transcribed and amplified by PCR. Liver samples with the highest S-mephenytoin hydroxylase activity contained only the normally spliced mRNA. By contrast, sample 35 (a probable poor metabolizer) produced an amplification product containing the 40 bp deletion. Samples with intermediate S-mephenytoin 4'-hydroxylase activity and low amounts of CYP2C19 protein exhibited both the normal 2C19 cDNA and 2C19 cDNA containing the 40 bp deletion.

15 (c) Genomic Sequencing of 2C19

Because human tissue samples containing genomic 2C19 DNA are much more easily obtained than samples containing 2C19 mRNA, it is preferable to diagnose a polymorphic defect from genomic DNA. Genomic DNA was isolated from the blood of human volunteers previously characterized as poor or extensive metabolizers of S-mephenytoin *in vivo*. The *in vivo* phenotype of most Swiss subjects was based on a hydroxylation index, with a value above 5.6 identifying a poor metabolizer (Kupfer et al., *Eur. J. Clin. Pharmacol.* 26:753-759 (1984)). The *in vivo* phenotype of American, Oriental and one Swiss subject was based on the urinary S/R ratio (Wedlund et al., *Clin. Pharmacol. Ther.* 36:773-780 (1984))--a poor metabolizer (PM) being defined as having a ratio > 0.95. An extensive metabolizer is defined as having a ratio < 0.8. An intermediate phenotype (IM) has been previously described with the extent of 4'-hydroxylation being greater than in PMS but with the rate of metabolite formation being slower than EMS (Arns et al., *Pharmacologist* 32:140 (1990)).

It was believed that the 40 bp deletion identified in 2C19 cDNA occurred in exon 5, near the border with intron 4 based on a comparison of the gene structure of CYP2C9 and CYP2C18 (de Morais et al., *supra*). Thus, a segment of genomic

2C19 DNA across the intron 4/exon 5 border was amplified to identify the corresponding genetic defect in genomic DNA. In the initial assays, the untranslated regions of the genomic 2C19 sequence were not known. However, intron 4 primers could be designed based on the corresponding sequences from CYP2C9, which are expected to show about 95% sequence identity based on comparison with partial genomic sequences of 2C19. The primer for exon 5 was based on the cDNA sequence of CYP2C19 (see Example 1). The amplified DNA fragment was found to have the same size in both poor and extensive metabolizers. However, on restriction analysis, it was found that only the fragment from extensive metabolizers could be digested with SmaI. The amplified DNA fragment was sequenced in extensive and poor metabolizers.

Provision of genomic 2C19 DNA sequence in the intron 4 region, allowed the design of a specific intron primer exhibiting perfect complementarity to the 2C19 DNA sequence in subsequent experiments. The forward PCR primer from intron 4 was 5'-AATTACAACCAGAGCTTGGC-3' and the reverse primer from exon 5 was 5'-TATCACTTTCCATAAAAGCAAG-3'. The forward primer anneals 81 bp upstream of the intron 4/exon 5 junction. PCR conditions were as for amplification of cDNA except that reactions used 200 ng of genomic DNA and an initial denaturation at 96°C for 5 min. PCR products were restricted with SmaI in the PCR buffer, without purification. Uncut products had the same size (168 bp) in all samples. Digested PCR products were analyzed on 4% agarose gels stained with ethidium bromide.

DNA from 18 unrelated Caucasian extensive metabolizers and 10 unrelated Caucasian poor metabolizers was analyzed by this strategy. (Fig 14C). All extensive metabolizers were either homozygous or heterozygous for the normal CYP2C19 gene, defined here as CYP2C19_{wt} (wild type). Among the 10 poor metabolizers, 7 were homozygous for the defective gene, defined as CYP2C19_m (poor mephenytoin hydroxylation). One poor metabolizer was heterozygous (CYP2C19_{wt}/CYP2C19_m), and two were homozygous (CYP2C19_{wt}/CYP2C19_{wt}), indicating that CYP2C19_m accounted for

15 of 20 alleles tested (75%) in Caucasian poor metabolizers. The presence of 5 CYP2C19_{wt} alleles in poor metabolizers suggests that additional mutations may exist in the Caucasian population, but that 2C19_m represents the predominant defect.

5 Segments of DNA spanning the intron 4/exon 5 boundary were also amplified from 17 unrelated Oriental subjects. Figure 14D shows that 10/17 Oriental poor metabolizers are homozygous for CYP2C19_m, and CYP2C19_m accounts for 25 of 34 alleles (74%) in Oriental poor
10 metabolizers. All 12 unrelated Oriental extensive metabolizers were either homozygous or heterozygous for the CYP2C19_{wt} gene. Thus, the major mutation responsible for the poor metabolizer phenotype in Oriental is identical to that found in Caucasians.

15 The inheritance of CYP2C19_m in one Oriental family previously characterized with respect to the PM trait was also examined. Figure 14B shows that the poor metabolizer proband (arrow) and two other related poor metabolizers are homozygous for CYP2C19_m. Two individuals identified earlier as obligate
20 heterozygotes (family C) (Ward et al., *Clin. Pharmacol. Ther.* 42:96-99 (1987)) were indeed found to be CYP2C19_m/CYP2C19_{wt}. Thus, the inheritance of the genotype agrees with the Mendelian autosomal-recessive inheritance of phenotype.

 The DNA of three individuals (CYP2C19_{wt}/CYP2C19_{wt},
25 CYP2C19_m/CYP2C19_m, and CYP2C19_{wt}/CYP2C19_m) was amplified as described above and sequenced directly using an automated sequencer (Applied Biosystems) (Fig. 15). Surprisingly, the sequence of intron 4 of the defective gene was identical to that of the normal gene. The only alteration found in
30 CYP2C19_m was a G→A change in exon 5 corresponding to position 681 of the cDNA. This mutation introduces a cryptic splice site in this exon. This mutation also abolishes a SmaI site at this position (CCCGGG → CCCAGG). The cryptic splice site shows slightly greater sequence identity to the consensus
35 sequence for mammalian splice sites (Green, *Ann. Rev. Cell Biol.* 7:559-599 (1991)) than the normal splice site. A second potential branch point is also seen near the cryptic splice site. Surprisingly, the cDNA sequences from CYP2C8 and

CYP2C18 have a comparable potential cryptic splice site at the same point in exon 5 to that of CYP2C19_m, but the presence of the full-length 2C8 protein on immunoblots of human liver microsomes indicates that the majority of this protein is spliced correctly.

Three of the samples tested by cDNA analysis in Figure 13 (sample 13, predicted genotype CYP2C19_{wt}/CYP2C19_{wt}), sample 21, predicted genotype CYP2C19_{wt}/CYP2C19_m, and sample 35, predicted genotype CYP2C19_m/CYP2C19_m) were retested by genomic analysis. Perfect agreement was observed. The cryptic splice site appeared to be used exclusively in sample 35 which is a predicted poor metabolizer and also in liver RNA of an additional CYP2C19_m/CYP2C19_m individual. The selection of the cryptic splice site results in the absence of CYP2C19 in liver microsomes from poor metabolizers (Fig. 13).

(d) Conclusion

The principal genetic defect (CYP2C19_m) which is responsible for the poor metabolism of S-mephenytoin is a G-A mutation at position 681 of the coding sequence (within exon 5). CYP2C19_m accounts for 75% of the defective alleles in both Caucasian and Oriental poor metabolizers. The single base change generates a cryptic internal splice site, which is used exclusively to produce an aberrantly spliced mRNA containing a 40 bp deletion. The CYP2C19 protein is virtually absent in livers of poor metabolizers. The mutation at position 681 is easily detected by PCR amplification of a segment of genomic 2C19 DNA spanning the mutation.

Example 7: Identification and Diagnostic Assay for a Second Polymorphism (designated 636) in 2C19

A second mutation designated the 636 polymorphism (also known as CYP2C19_{m2}) has identified. Genomic DNA from a Oriental poor metabolizer (subject 43 in Example 6) was amplified by PCR using a forward primer complementary to the antisense strand of intron 3 extending from bases -79 to -55 and a reverse primer complementary to the sense strand extending from 79-89 bases into intron 4 (forward primer 5'-

TATTATCTGTAACTAATATGA-3' (SEQ. ID. No. 57) and reverse primer 5'- ACTTCAGGGCTTGGTCAATA-3' (SEQ. ID. No. 58). These primers were selected to amplify a 329 base pair product containing all of exon 4 and the surrounding intron/exon junctions. See Figure 17. Sequencing of the PCR products with an Applied Biosystems sequencer identified two mutations in exon 4 of the Oriental poor metabolizer. A second mutation at nucleotide 636 entailed a G→A transition at the nucleotide level and the conversion of a tryptophan codon at position 212 (TGG→TGA) to a premature stop codon. This change would result in a truncated 211 amino acid polypeptide containing only the first 4 exons, which would not contain the heme-binding region and would be inactive. The change at position 636 also destroys a BamHI site (GGATCC→GAATCC) (or its isoschizomer BstI) at positions 635-640.

A PCR test was developed using the primers described above to amplify a 329 base pair product. The PCR product from the wild-type DNA from extensive metabolizers was cut with BamHI to yield two expected fragments with sizes of 233 base pairs and 96 base pairs (Fig. 18). The PCR fragment amplified from the individual with the 636 mutation (i.e., Oriental subject #43) could not be restricted, indicating that he was homozygous for the 636 mutation. Genotyping of 7 Oriental poor metabolizers whose phenotype could not be explained by the previous 681 mutation indicated that subjects 41 and 43 were homozygous for the 636 mutation, while subjects 36, 48, 11, 69, and 100, were heterozygous for bearing both 636 and 681 mutant alleles. The DNA in homozygous 636 mutant subjects 41 and 43 was not cut by BamHI. The DNA in the heterozygotes yielded three bands at 327, 232, and 95 bp. The DNA from these heterozygotes also yielded three bands from SmaI site (169, 120, and 49 bp) indicating they were also heterozygous for the 681 base pair mutation named CYP2C19_m). These data show that the 636 and 681 mutations completely account for the low phenotypes in all of the Oriental poor metabolizers of S-mephenytoin tested (17 individuals with 34 alleles).

Three Caucasian poor metabolizers who were not homozygous for the 681 mutation were also genotyped for the 636 mutation. These were subjects JOB1, 502 and 503. One of these individuals (JOB1) was heterozygous for the 681 mutation while the other two did not contain the 681 mutation in either allele. None of these individuals exhibited a 636 mutation. Thus, there is probably at least one additional polymorphism in 2C19 in Caucasians.

In summary, the 681 and 636 mutations explain 100% of Oriental poor metabolizers, and the 681 mutation alone accounts for about 75% of Caucasian poor metabolizers.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: GOLDSTEIN, Joyce A.
ROMKES-SPARKS, Marjorie
DE MORAIS, Sonia M.F.
- (ii) TITLE OF INVENTION: CLONING, EXPRESSION AND DIAGNOSIS OF
HUMAN CYTOCHROME P450 2C19: THE PRINCIPAL
DETERMINANT OF S-MEPHINYTOIN METABOLISM
- (iii) NUMBER OF SEQUENCES: 61
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Townsend and Townsend Khourie and Crew
 - (B) STREET: 379 Lytton Avenue
 - (C) CITY: Palo Alto
 - (D) STATE: California
 - (E) COUNTRY: US
 - (F) ZIP: 94301
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/238,821
 - (B) FILING DATE: 06-MAY-1994
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 08/201,118
 - (B) FILING DATE: 22-FEB-1994
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: US 07/864,962
 - (B) FILING DATE: 09-APR-1992
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Dow, Karen B.
 - (B) REGISTRATION NUMBER: 29,684
 - (C) REFERENCE/DOCKET NUMBER: 15280-192-1-1
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (415) 326-2400
 - (B) TELEFAX: (415) 326-2422

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 490 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Met Asp Pro Phe Val Val Leu Val Leu Cys Leu Ser Cys Leu Leu Leu
 1              5              10              15

Leu Ser Ile Trp Arg Gln Ser Ser Gly Arg Gly Lys Leu Pro Pro Gly
 20              25              30

Pro Thr Pro Leu Pro Val Ile Gly Asn Ile Leu Gln Ile Asp Ile Lys
 35              40              45

Asp Val Ser Lys Ser Leu Thr Asn Leu Ser Lys Ile Tyr Gly Pro Val
 50              55              60

Phe Thr Leu Tyr Phe Gly Leu Glu Arg Met Val Val Leu His Gly Tyr
 65              70              75              80

Glu Val Val Lys Glu Ala Leu Ile Asp Leu Gly Glu Glu Phe Ser Gly
 85              90              95

Arg Gly His Phe Pro Leu Ala Glu Arg Ala Asn Arg Gly Phe Gly Ile
100              105              110

Val Phe Ser Asn Gly Lys Arg Trp Lys Glu Ile Arg Arg Phe Ser Leu
115              120              125

Met Thr Leu Arg Asn Phe Gly Met Gly Lys Arg Ser Ile Glu Asp Arg
130              135              140

Val Gln Glu Glu Ala Arg Cys Leu Val Glu Glu Leu Arg Lys Thr Lys
145              150              155              160

Ala Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn
165              170              175

Val Ile Cys Ser Ile Ile Phe Gln Lys Arg Phe Asp Tyr Lys Asp Gln
180              185              190

Gln Phe Leu Asn Leu Met Glu Lys Leu Asn Glu Asn Ile Arg Ile Val
195              200              205

Ser Thr Pro Trp Ile Gln Ile Cys Asn Asn Phe Pro Thr Ile Ile Asp
210              215              220

Tyr Phe Pro Gly Thr His Asn Lys Leu Leu Lys Asn Leu Ala Phe Met
225              230              235              240

Glu Ser Asp Ile Leu Glu Lys Val Lys Glu His Gln Glu Ser Met Asp
245              250              255

Ile Asn Asn Pro Arg Asp Phe Ile Asp Cys Phe Leu Ile Lys Met Glu
260              265              270

Lys Glu Lys Gln Asn Gln Gln Ser Glu Phe Thr Ile Glu Asn Leu Val
275              280              285

Ile Thr Ala Ala Asp Leu Leu Gly Ala Gly Thr Glu Thr Thr Ser Thr
290              295              300

```


91

Thr Leu Arg Tyr Ala Leu Leu Leu Leu Leu Lys His Pro Glu Val Thr
 305 310 315 320
 Ala Lys Val Gln Glu Glu Ile Glu Arg Val Ile Gly Arg Asn Arg Ser
 325 330 335
 Pro Cys Met Gln Asp Arg Gly His Met Pro Tyr Thr Asp Ala Val Val
 340 345 350
 His Glu Val Gln Arg Tyr Ile Asp Leu Ile Pro Thr Ser Leu Pro His
 355 360 365
 Ala Val Thr Cys Asp Val Lys Phe Arg Asn Tyr Leu Ile Pro Lys Gly
 370 375 380
 Thr Thr Ile Leu Thr Ser Leu Thr Ser Val Leu His Asp Asn Lys Glu
 385 390 395 400
 Phe Pro Asn Pro Glu Met Phe Asp Pro Arg His Phe Leu Asp Glu Gly
 405 410 415
 Gly Asn Phe Lys Lys Ser Asn Tyr Phe Met Pro Phe Ser Ala Gly Lys
 420 425 430
 Arg Ile Cys Val Gly Glu Gly Leu Ala Arg Met Glu Leu Phe Leu Phe
 435 440 445
 Leu Thr Phe Ile Leu Gln Asn Phe Asn Leu Lys Ser Leu Ile Asp Pro
 450 455 460
 Lys Asp Leu Asp Thr Thr Pro Val Val Asn Gly Phe Ala Ser Val Pro
 465 470 475 480
 Pro Phe Tyr Gln Leu Cys Phe Ile Pro Val
 485 490

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1746 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTCAATGGA TCCTTTTGTG GTCCTTGTGC TCTGTCTCTC ATGTTTGCTT CTCCTTTCAA	60
TCTGGAGACA GAGCTCTGGG AGAGGAAAAC TCCCTCCTGG CCCCCTCCT CTCCAGTGA	120
TTGGAAATAT CCTACAGATA GATATTAAGG ATGTCAGCAA ATCCTTAACC AATCTCTCAA	180
AAATCTATGG CCCTGTGTTT ACTCTGTATT TTGGCCTGGA ACGCATGGTG GTGCTGCATG	240
GATATGAAGT GGTGAAGGAA GCCCTGATTG ATCTTGGAGA GGAGTTTTCT GGAAGAGGCC	300
ATTTCCCACT GGCTGAAAGA GCTAACAGAG GATTTGGAAT CGTTTTTCAGC AATGGAAAGA	360
GATGGAAGGA GATCCGGCGT TTCTCCCTCA TGACGCTGCG GAATTTTGGG ATGGGGAAGA	420
GGAGCATTGA GGACCGTGTT CAAGAGGAAG CCCGCTGCCT TGTGGAGGAG TTGAGAAAAA	480
CCAAGGCTTC ACCCTGTGAT CCCACTTTCA TCCTGGGCTG TGCTCCCTGC AATGTGATCT	540
GCTCCATTAT TTTCCAGAAA CGTTTCGATT ATAAAGATCA GCAATTTCTT AACTTGATGG	600
AAAAATTGAA TGAAAACATC AGGATTGTAA GCACCCCTG GATCCAGATA TGCAATAATT	660
TTCCCACTAT CATTGATTAT TTCCCGGGAA CCCATAACAA ATTACTTAAA AACCTTGCTT	720
TTATGGAAAG TGATATTTTG GAGAAAGTAA AAGAACACCA AGAATCGATG GACATCAACA	780
ACCCTCGGGA CTTTATTGAT TGCTTCCTGA TCAAAATGGA GAAGGAAAAG CAAAACCAAC	840
AGTCTGAATT CACTATTGAA AACTTGGTAA TCACTGCAGC TGACTTACTT GGAGCTGGGA	900
CAGAGACAAC AAGCACAACC CTGAGATATG CTCTCCTTCT CCTGCTGAAG CACCCAGAGG	960
TCACAGCTAA AGTCCAGGAA GAGATTGAAC GTGTCAATTG CAGAAACCGG AGCCCCTGCA	1020
TGCAGGACAG GGGCCACATG CCTACACAG ATGCTGTGGT GCACGAGGTC CAGAGATACA	1080
TCGACCTCAT CCCACACAG CTGCCCCATG CAGTGACCTG TGACGTTAAA TTCAGAAACT	1140

```

ACCTCATTCC CAAGGGCACA ACCATATTAA CTTCCCTCAC TTCTGTGCTA CATGACAACA      1200
AAGAATTTCC CAACCCAGAG ATGTTTGACC CTCGTCACCTT TCTGGATGAA GGTGGAAATT      1260
TTAAGAAAAG TAACTACTTC ATGCCTTTCT CAGCAGGAAA ACGGATTTGT GTGGGAGAGG      1320
GCCTGGCCCG CATGGAGCTG TTTTATTTC TGACCTTCAT TTTACAGAAC TTAAACCTGA      1380
AATCTCTGAT TGACCCAAAG GACCTTGACA CAACTCCTGT TGTCAATGGA TTTGCTTCTG      1440
TCCCGCCCTT CTATCAGCTG TGCTTCATTC CTGTCTGAAG AAGCACAGAT GGTCTGGCTG      1500
CTCCTGTGCT GTCCCTGCAG CTCTCTTTCC TCTGGTCCAA ATTTCACTAT CTGTGATGCT      1560
TCTTCTGACC CGTCATCTCA CATTTTCCCT TCCCCAAGA TCTAGTGAAC ATTCAGCCTC      1620
CATTAAAAAA GTTTCACTGT GCAAATATAT CTGCTATTCC CCATACTCTA TAATAGTTAC      1680
ATTGAGTGCC ACATAATGCT GATACTTGTC TAATGTTGAG TTATTAACAT ATTATTATTA      1740
AATAGA                                          1746

```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 490 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

Met Asp Ser Leu Val Val Leu Val Leu Cys Leu Ser Cys Leu Leu Leu      1
1          5          10          15
Leu Ser Leu Trp Arg Gln Ser Ser Gly Arg Gly Lys Leu Pro Pro Gly
20          25          30
Pro Thr Pro Leu Pro Val Ile Gly Asn Ile Leu Gln Ile Gly Ile Lys
35          40          45
Asp Ile Ser Lys Ser Leu Thr Asn Leu Ser Lys Val Tyr Gly Pro Val
50          55          60
Phe Thr Leu Tyr Phe Gly Leu Lys Pro Ile Val Val Leu His Gly Tyr
65          70          75          80
Glu Ala Val Lys Glu Ala Leu Ile Asp Leu Gly Glu Glu Phe Ser Gly
85          90          95
Arg Gly Ile Phe Pro Leu Ala Glu Arg Ala Asn Arg Gly Phe Gly Ile
100         105         110
Val Phe Ser Asn Gly Lys Lys Trp Lys Glu Ile Arg Arg Phe Ser Leu
115         120         125
Met Thr Leu Arg Asn Phe Gly Met Gly Lys Arg Ser Ile Glu Asp Arg
130         135         140
Val Gln Glu Glu Ala Arg Cys Leu Val Glu Glu Leu Arg Lys Thr Lys
145         150         155         160
Ala Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn
165         170         175

```

Val Ile Cys Ser Ile Ile Phe His Lys Arg Phe Asp Tyr Lys Asp Gln
 180 185 190
 Gln Phe Leu Asn Leu Met Glu Lys Leu Asn Glu Asn Ile Lys Ile Leu
 195 200 205
 Ser Ser Pro Trp Ile Gln Ile Cys Asn Asn Phe Ser Pro Ile Ile Asp
 210 215 220
 Tyr Phe Pro Gly Thr His Asn Lys Leu Leu Lys Asn Val Ala Phe Met
 225 230 235 240
 Lys Ser Tyr Ile Leu Glu Lys Val Lys Glu His Gln Glu Ser Met Asp
 245 250 255
 Met Asn Asn Pro Gln Asp Phe Ile Asp Cys Phe Leu Met Lys Met Glu
 260 265 270
 Lys Glu Lys His Asn Gln Pro Ser Glu Phe Thr Ile Glu Ser Leu Glu
 275 280 285
 Asn Thr Ala Val Asp Leu Phe Gly Ala Gly Thr Glu Thr Thr Ser Thr
 290 295 300
 Thr Leu Arg Tyr Ala Leu Leu Leu Leu Lys His Pro Glu Val Thr
 305 310 315 320
 Ala Lys Val Gln Glu Glu Ile Glu Arg Val Ile Gly Arg Asn Arg Ser
 325 330 335
 Pro Cys Met Gln Asp Arg Ser His Met Pro Tyr Thr Asp Ala Val Val
 340 345 350
 His Glu Val Gln Arg Tyr Leu Asp Leu Leu Pro Thr Ser Leu Pro His
 355 360 365
 Ala Val Thr Cys Asp Ile Lys Phe Arg Asn Tyr Leu Ile Pro Lys Gly
 370 375 380
 Thr Thr Ile Leu Ile Ser Leu Thr Ser Val Leu His Asp Asn Lys Glu
 385 390 395 400
 Phe Pro Asn Pro Glu Met Phe Asp Pro His His Phe Leu Asp Glu Gly
 405 410 415
 Gly Asn Phe Lys Lys Ser Lys Tyr Phe Met Pro Phe Ser Ala Gly Lys
 420 425 430
 Arg Ile Cys Val Gly Glu Ala Leu Ala Gly Met Glu Leu Phe Leu Phe
 435 440 445
 Leu Thr Ser Ile Leu Gln Asn Phe Asn Leu Lys Ser Leu Val Asp Pro
 450 455 460
 Lys Asn Leu Asp Thr Thr Pro Val Val Asn Gly Phe Ala Ser Val Pro
 465 470 475 480
 Pro Phe Tyr Gln Leu Cys Phe Ile Pro Val
 485 490

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 1854 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGAAGGCTT CAATGGATTC TCTTGTGGTC CTTGTGCTCT GTCTCTCATG TTTGCTTCTC	60
CTTTCACCTCT GGAGACAGAG CTCTGGGAGA GGAAAACTCC CTCCTGGCCC CACTCCTCTC	120
CCAGTGATTG GAAATATCCT ACAGATAGGT ATTAAGGACA TCAGCAAATC CTTAACCAAT	180
CTCTCAAAGG TCTATGGCCC TGTGTTCACT CTGTATTTTG GCCTGAAACC CATAGTGGTG	240
CTGCATGGAT ATGAAGCAGT GAAGGAAGCC CTGATTGATC TTGGAGAGGA GTTTTCTGGA	300
AGAGGCATTT TCCCCTGGC TGAAAGAGCT AACAGAGGAT TTGGAATTGT TTTCAGCAAT	360
GGAAAGAAAT GGAAGGAGAT CCGGCGTTTC TCCCTCATGA CGCTGCGGAA TTTTGGGATG	420
GGGAAGAGGA GCATTGAGGA CCGTGTTCAA GAGGAAGCCC GCTGCCTTGT GGAGGAGTTG	480
AGAAAAACCA AGGCCTCACC CTGTGATCCC ACTTTCATCC TGGGCTGTGC TCCCTGCAAT	540
GTGATCTGCT CCATTATTTT CCATAAACGT TTTGATTATA AAGATCAGCA ATTTCTTAAC	600
TTAATGGAAA AGTTGAATGA AAACATCAAG ATTTTGAGCA GCCCCTGGAT CCAGATCTGC	660
AATAATTTTT CTCCTATCAT TGATTACTTC CCGGGAAGTC ACAACAAATT ACTTAAAAAC	720
GTTGCTTTTA TGAAAAGTTA TATTTTGGA AAAGTAAAG AACACCAAGA ATCAATGGAC	780
ATGAACAACC CTCAGGACTT TATTGATTGC TTCCTGATGA AAATGGAGAA GGAAAAGCAC	840
AACCAACCAT CTGAATTAC TATTGAAAGC TTGGAAAACA CTGCAGTTGA CTTGTTTGGA	900
GCTGGGACAG AGACGACAAG CACAACCCTG AGATATGCTC TCCTTCTCCT GCTGAAGCAC	960
CCAGAGGTCA CAGCTAAAGT CCAGGAAGAG ATTGAACGTG TGATTGGCAG AAACCGGAGC	1020
CCCTGCATGC AAGACAGGAG CCACATGCCC TACACAGATG CTGTGGTGCA CGAGGTCCAG	1080
AGATACCTTG ACCTTCTCCC CACCAGCCTG CCCCATGCAG TGACCTGTGA CATTAATTC	1140
AGAACTATC TCATTCCCAA GGGCACAACC ATATTAATTT CCCTGACTTC TGTGCTACAT	1200
GACAACAAAG AATTTCCCAA CCCAGAGATG TTTGACCCTC ATCACTTTCT GGATGAAGGT	1260
GGCAATTTTA AGAAAAGTAA ATACTTCATG CCTTCTCAG CAGGAAAACG GATTTGTGTG	1320
GGAGAAGCCC TGGCCGGCAT GGAGCTGTTT TTATTCCTGA CCTCCATTTT ACAGAACTTT	1380
AACCTGAAAT CTCTGGTTGA CCCAAAGAAC CTTGACACCA CTCCAGTTGT CAATGGTTTT	1440
GCCTCTGTGC CGCCCTTCTA CCAGCTGTGC TTCATTCCTG TCTGAAGAAG AGCAGATGGC	1500
CTGGCTGCTG CTGTGCAGTC CCTGCAGCTC TCTTCTCTCT GGGGCATTAT CCATCTTTCA	1560
CTATCTGTAA TGCCTTTTCT CACCTGTCAT CTCACATTTT CCCTTCCCTG AAGATCTAGT	1620
GAACATTCGA CCTTCATTAC GGAGAGTTTC CTATGTTTCA CTGTGCAAAT ATATCTGCTA	1680

TTCTCCATAC TCTGTAACAG TTGCATTGAC TGTCACATAA TGCTCATACT TATCTAATGT 1740
 TGAGTTATTA ATATGTTATT ATTAAATAGA GAAATATGAT TTGTGTATTA TAATTCAAAG 1800
 GCATTTCTTT TCTGCATGTT CTAAATAAAA AGCATTATTA TTTGCTGAAA AAAA 1854

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 490 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Asp Pro Ala Val Ala Leu Val Leu Cys Leu Ser Cys Leu Phe Leu
 1 5 10 15
 Leu Ser Leu Trp Arg Gln Ser Ser Gly Arg Gly Arg Leu Pro Ser Gly
 20 25 30
 Pro Thr Pro Leu Pro Ile Ile Gly Asn Ile Leu Gln Leu Asp Val Lys
 35 40 45
 Asp Met Ser Lys Ser Leu Thr Asn Phe Ser Lys Val Tyr Gly Pro Val
 50 55 60
 Phe Thr Val Tyr Phe Gly Leu Lys Pro Ile Val Val Leu His Gly Tyr
 65 70 75 80
 Glu Ala Val Lys Glu Ala Leu Ile Asp His Gly Glu Glu Phe Ser Gly
 85 90 95
 Arg Gly Ser Phe Pro Val Ala Glu Lys Val Asn Lys Gly Leu Gly Ile
 100 105 110
 Leu Phe Ser Asn Gly Lys Arg Trp Lys Glu Ile Arg Arg Phe Cys Leu
 115 120 125
 Met Thr Leu Arg Asn Phe Gly Met Gly Lys Arg Ser Ile Glu Asp Arg
 130 135 140
 Val Gln Glu Glu Ala Arg Cys Leu Val Glu Glu Leu Arg Lys Thr Asn
 145 150 155 160
 Ala Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn
 165 170 175
 Val Ile Cys Ser Val Ile Phe His Asp Arg Phe Asp Tyr Lys Asp Gln
 180 185 190
 Arg Phe Leu Asn Leu Met Glu Lys Phe Asn Glu Asn Leu Arg Ile Leu
 195 200 205

Ser Ser Pro Trp Ile Gln Val Cys Asn Asn Phe Pro Ala Leu Ile Asp
 210 215 220
 Tyr Leu Pro Gly Ser His Asn Lys Ile Ala Glu Asn Phe Ala Tyr Ile
 225 230 235 240
 Lys Ser Tyr Val Leu Glu Arg Ile Lys Glu His Gln Glu Ser Leu Asp
 245 250 255
 Met Asn Ser Ala Arg Asp Phe Ile Asp Cys Phe Leu Ile Lys Met Glu
 260 265 270
 Gln Glu Lys His Asn Gln Gln Ser Glu Phe Thr Val Glu Ser Leu Ile
 275 280 285
 Ala Thr Val Thr Asp Met Phe Gly Ala Gly Thr Glu Thr Thr Ser Thr
 290 295 300
 Thr Leu Arg Tyr Gly Leu Leu Leu Leu Lys Tyr Pro Glu Val Thr
 305 310 315 320
 Ala Lys Val Gln Glu Glu Ile Glu Cys Val Val Gly Arg Asn Arg Ser
 325 330 335
 Pro Cys Met Gln Asp Arg Ser His Met Pro Tyr Thr Asp Ala Val Val
 340 345 350
 His Glu Ile Gln Arg Tyr Ile Asp Leu Leu Pro Thr Asn Leu Pro His
 355 360 365
 Ala Val Thr Cys Asp Val Lys Phe Lys Asn Tyr Leu Ile Pro Lys Gly
 370 375 380
 Thr Thr Ile Ile Thr Ser Leu Thr Ser Val Leu His Asn Asp Lys Glu
 385 390 395 400
 Phe Pro Asn Pro Glu Met Phe Asp Pro Gly His Phe Leu Asp Lys Ser
 405 410 415
 Gly Asn Phe Lys Lys Ser Asp Tyr Phe Met Pro Phe Ser Ala Gly Lys
 420 425 430
 Arg Met Cys Met Gly Glu Gly Leu Ala Arg Met Glu Leu Phe Leu Phe
 435 440 445
 Leu Thr Thr Ile Leu Gln Asn Phe Asn Leu Lys Ser Gln Val Asp Pro
 450 455 460
 Lys Asp Ile Asp Ile Thr Pro Ile Ala Asn Ala Phe Gly Arg Val Pro
 465 470 475 480

Pro Leu Tyr Gln Leu Cys Phe Ile Pro Val
485 490

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2009 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGCACCGGAA AGAACAAGAA AAAAGAACAC CTTATTTTAA TCTTCTTCAG TGAGCCAATG	60
TTCATTCAAA AGAGAGATTA AAGTGCTTTT TGCTGACTAG TCACAGTCAG AGTCAGAATC	120
ACAGGTGGAT TAGTAGGGAG TGTATAAAAA GCCTTGAAGT GAAAGCCCGC AGTTGTCTTA	180
CTAAGAAGAG AAGCCTTCAA TGGATCCAGC TGTGGCTCTG GTGCTCTGTC TCTCCTGTTT	240
GTTTCTCCTT TCACTCTGGA GGCAGAGCTC TGGGAAGAGG AGGCTCCCGT CTGGCCCCAC	300
TCCTCTCCCG ATTATTGGAA ATATCCTGCA GTTAGATGTT AAGGACATGA GCAAATCCTT	360
AACCAATTTT TCAAAAGTCT ATGGCCCTGT GTTCACTGTG TATTTTGGCC TGAAGCCCAT	420
TGTGGTGTG CATGGATATG AAGCAGTGAA GGAGGCCCTG ATTGATCATG GAGAGGAGTT	480
TTCTGGAAGA GGAAGTTTTC CAGTGGCTGA AAAAGTTAAC AAAGGACTTG GAATCCTTTT	540
CAGCAATGGA AAGAGATGGA AGGAGATCCG GCGTTTCTGC CTCATGACTC TCGGGAATTT	600
TGGGATGGGG AAGAGGAGCA TCGAGGACCG TGTTCAAGAG GAAGCCCGCT GCCTTGTGGA	660
GGAGTTGAGA AAAACCAATG CCTCACCTG TGATCCCACT TTCATCCTGG GCTGTGCTCC	720
CTGCAATGTG ATCTGCTCTG TTATTTTCCA TGATCGATTT GATTATAAAG ATCAGAGGTT	780
TCTTAACCTG ATGGAAAAAT TCAATGAAAA CCTCAGGATT CTGAGCTCTC CATGGATCCA	840
GGTCTGCAAT AATTTCCCTG CTCTCATCGA TTATCTCCCA GGAAGTCATA ATAAATAGC	900
TGAAAATTTT GCTTACATTA AAAGTTATGT ATTGGAGAGA ATAAAAGAAC ATCAAGAATC	960
CCTGGACATG AACAGTGCTC GGGACTTTAT TGATTGTTTC CTGATCAAAA TGGAACAGGA	1020
AAAGCACAAT CAACAGTCTG AATTTACTGT TGAAAGCTTG ATAGCCACTG TAACTGATAT	1080
GTTTGGGGCT GGAACAGAGA CAACGAGCAC CACTCTGAGA TATGGACTCC TGCTCCTGCT	1140
GAAGTACCCA GAGGTACAG CTAAAGTCCA GGAAGAGATT GAATGTGTAG TTGGCAGAAA	1200
CCGGAGCCCC TGTATGCAGG ACAGGAGTCA CATGCCCTAC ACAGATGCTG TGGTGCACGA	1260
GATCCAGAGA TACATTGACC TCCTCCCCAC CAACCTGCCC CATGCAGTGA CCTGTGATGT	1320
TAAATTCAA AACTACCTCA TCCCCAAGGG CACGACCATA ATAACATCCC TGACTTCTGT	1380
GCTGCACAAT GACAAAGAAT TCCCCAACCC AGAGATGTTT GACCCTGGCC ACTTTCTGGA	1440
TAAGAGTGGC AACTTTAAGA AAAGTGAATA CTTTCATGCCT TTCTCAGCAG GAAAACGGAT	1500
GTGTATGGGA GAGGGCCTGG CCCGCATGGA GCTGTTTTTA TTCCTGACCA CCATTTTGCA	1560


```

GAACTTTAAC CTGAAATCTC AGGTTGACCC AAAGGATATT GACATCACCC CCATTGCCAA      1620
TGCATTTGGT CGTGTGCCAC CCTTGTACCA GCTCTGCTTC ATTCTGTCTT GAAGAAGGGC      1680
AGATAGTTTG GCTGCTCCTG TGCTGTCACC TGCAATTCTC CCTTATCAGG GCCATTAGCC      1740
TCTCCCTTCT CTCTGTGAGG GATATTTTCT CTGACTTGTC AATCCACATC TTCCCATTCC      1800
CTCAAGATCC AATGAACATC CAACCTCCAT TAAAGAGAGT TTCTTGGGTC ACTTCCTAAA      1860
TATATCTGCT ATTCTCCATA CTCTGTATCA CTTGTATTGA CCACCACATA TGCTAATACC      1920
TATCTACTGC TGAGTTGTCA GTATGTTATC ACTAGAAAAC AAAGAAAAAT GATTAATAAA      1980
TGACAATTCA GAGCCAAAAA AAAAAAAAAA      2009

```

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 490 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Met Glu Pro Phe Val Val Leu Val Leu Cys Leu Ser Phe Met Leu Leu
1           5           10           15
Phe Ser Leu Trp Arg Gln Ser Cys Arg Arg Arg Lys Leu Pro Pro Gly
20          25          30
Pro Thr Pro Leu Pro Ile Ile Gly Asn Met Leu Gln Ile Asp Val Lys
35          40          45
Asp Ile Cys Lys Ser Phe Thr Asn Phe Ser Lys Val Tyr Gly Pro Val
50          55          60
Phe Thr Val Tyr Phe Gly Met Asn Pro Ile Val Val Phe His Gly Tyr
65          70          75          80
Glu Ala Val Lys Glu Ala Leu Ile Asp Asn Gly Glu Glu Phe Ser Gly
85          90          95
Arg Gly Asn Ser Pro Ile Ser Gln Arg Ile Thr Lys Gly Leu Gly Ile
100         105         110
Ile Ser Ser Asn Gly Lys Arg Trp Lys Glu Ile Arg Arg Phe Ser Leu
115         120         125
Thr Asn Leu Arg Asn Phe Gly Met Gly Lys Arg Ser Ile Glu Asp Arg
130         135         140
Val Gln Glu Glu Ala His Cys Leu Val Glu Glu Leu Arg Lys Thr Lys
145         150         155         160
Ala Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn
165         170         175
Val Ile Cys Ser Val Val Phe Gln Lys Arg Phe Asp Tyr Lys Asp Gln
180         185         190

```

100

Asn Phe Leu Thr Leu Met Lys Arg Phe Asn Glu Asn Phe Arg Ile Leu
 195 200 205
 Asn Ser Pro Trp Ile Gln Val Cys Asn Asn Phe Pro Leu Leu Ile Asp
 210 215 220
 Cys Phe Pro Gly Thr His Asn Lys Val Leu Lys Asn Val Ala Leu Thr
 225 230 235 240
 Arg Ser Tyr Ile Arg Glu Lys Val Lys Glu His Gln Ala Ser Leu Asp
 245 250 255
 Val Asn Asn Pro Arg Asp Phe Met Asp Cys Phe Leu Ile Lys Met Glu
 260 265 270
 Gln Glu Lys Asp Asn Gln Lys Ser Glu Phe Asn Ile Glu Asn Leu Val
 275 280 285
 Gly Thr Val Ala Asp Leu Phe Val Ala Gly Thr Glu Thr Thr Ser Thr
 290 295 300
 Thr Leu Arg Tyr Gly Leu Leu Leu Leu Leu Lys His Pro Glu Val Thr
 305 310 315 320
 Ala Lys Val Gln Glu Glu Ile Asp His Val Ile Gly Arg His Arg Ser
 325 330 335
 Pro Cys Met Gln Asp Arg Ser His Met Pro Tyr Thr Asp Ala Val Val
 340 345 350
 His Glu Ile Gln Arg Tyr Ser Asp Leu Val Pro Thr Gly Val Pro His
 355 360 365
 Ala Val Thr Thr Asp Thr Lys Phe Arg Asn Tyr Leu Ile Pro Lys Gly
 370 375 380
 Thr Thr Ile Met Ala Leu Leu Thr Ser Val Leu His Asp Asp Lys Glu
 385 390 395 400
 Phe Pro Asn Pro Asn Ile Phe Asp Pro Gly His Phe Leu Asp Lys Asn
 405 410 415
 Gly Asn Phe Lys Lys Ser Asp Tyr Phe Met Pro Phe Ser Ala Gly Lys
 420 425 430
 Arg Ile Cys Ala Gly Glu Gly Leu Ala Arg Met Glu Leu Phe Leu Phe
 435 440 445
 Leu Thr Thr Ile Leu Gln Asn Phe Asn Leu Lys Ser Val Asp Asp Leu
 450 455 460

101

Lys Asn Leu Asn Thr Thr Ala Val Thr Lys Gly Ile Val Ser Leu Pro
 465 470 475 480

Pro Ser Tyr Gln Ile Cys Phe Ile Pro Val
 485 490

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1829 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AATGGAACCT TTTGTGGTCC TGGTGCTGTG TCTCTCTTTT ATGCTTCTCT TTTCACTCTG	60
GAGACAGAGC TGTAGGAGAA GGAAGCTCCC TCCTGGCCCC ACTCCTCTTC CTATTATTGG	120
AAATATGCTA CAGATAGATG TTAAGGACAT CTGCAAATCT TTCACCAATT TCTCAAAAGT	180
CTATGGTCCT GTGTTACCCG TGTATTTTGG CATGAATCCC ATAGTGGTGT TTCATGGATA	240
TGAGGCAGTG AAGGAAGCCC TGATTGATAA TGGAGAGGAG TTTTCTGGAA GAGGCAATTC	300
CCCAATATCT CAAAGAATTA CTAAAGGACT TGAATCATT TCCAGCAATG GAAAGAGATG	360
GAAGGAGATC CGGCGTTTCT CCCTCACAAA CTTGCGGAAT TTTGGGATGG GGAAGAGGAG	420
CATTGAGGAC CGTGTTCAAG AGGAAGCTCA CTGCCCTGTG GAGGAGTTGA GAAAAACCAA	480
GGCTTCACCC TGTGATCCCA CTTTCATCCT GGGCTGTGCT CCCTGCAATG TGATCTGCTC	540
CGTTGTTTTT CAGAAACGAT TTGATTATAA AGATCAGAAT TTTCTCACC TGATGAAAAG	600
ATTCAATGAA AACTTCAGGA TTCTGAACTC CCCATGGATC CAGGTCTGCA ATAATTTCCC	660
TCTACTCATT GATTGTTTCC CAGGAACTCA CAACAAAGTG CTTAAAAATG TTGCTCTTAC	720
ACGAAGTTAC ATTAGGGAGA AAGTAAAAGA ACACCAAGCA TCACTGGATG TTAACAATCC	780
TCGGGACTTT ATGGATTGCT TCCTGATCAA AATGGAGCAG GAAAAGGACA ACCAAAAGTC	840
AGAATTCAAT ATTGAAAAC TGGTTGGCAC TGTAGCTGAT CTATTTGTTG CTGGAACAGA	900
GACAACAAGC ACCACTCTGA GATATGGACT CCTGCTCCTG CTGAAGCACC CAGAGGTCAC	960
AGCTAAAGTC CAGGAAGAGA TTGATCATGT AATTGGCAGA CACAGGAGCC CCTGCATGCA	1020
GGATAGGAGC CACATGCCTT ACACTGATGC TGTAGTGCAC GAGATCCAGA GATACAGTGA	1080
CCTTGTCACC ACCGGTGTGC CCCATGCAGT GACCACTGAT ACTAAGTTCA GAAACTACCT	1140
CATCCCCAAG GGCACAACCA TAATGGCATT ACTGACTTCC GTGCTACATG ATGACAAAGA	1200
ATTTCTTAAT CCAAATATCT TTGACCCTGG CCCTTTCTA GATAAGAATG GCAACTTTAA	1260
GAAAAGTGAC TACTTCATGC CTTTCTCAGC AGGAAAACGA ATTTGTGCAG GAGAAGGACT	1320
TGCCCCGATG GAGCTATTTT TATTTCTAAC CACAATTTTA CAGAACTTTA ACCTGAAATC	1380
TGTTGATGAT TTAAAGAACC TCAATACTAC TGCAAGTTACC AAAGGGATTG TTTCTCTGCC	1440

102

ACCCTCATAC CAGATCTGCT TCATCCCTGT CTGAAGAATG CTAGCCCATC TGGCTGCTGA 1500
 TCTGCTATCA CCTGCAACTC TTTTITTTATC AAGGACATTC CCACTATTAT GTCTTCTCTG 1560
 ACCTCTCATC AAATCTTCCC ATTCACTCAA TATCCCATAA GCATCCAAAC TCCATTAAAG 1620
 AGAGTTGTTT AGGTCACTGC ACAAATATAT CTGCAATTAT TCATACTCTG TAACACTTGT 1680
 ATTAATTGCT GCATATGCTA ATACTTTTCT AATGCTGACT TTTTAATATG TTATCACTGT 1740
 AAAACACAGA AAAGTGATTA ATGAATGATA ATTTAGTCCA TTTCTTTTGT GAATGTGCTA 1800
 AATAAAAAGT GTTATTAAAT GCTGGTTCA 1829

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 490 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Met Asp Ser Leu Val Val Leu Val Leu Cys Leu Ser Cys Leu Leu Leu
 1 5 10 15
 Leu Ser Leu Trp Arg Gln Ser Ser Gly Arg Gly Lys Leu Pro Pro Gly
 20 25 30
 Pro Thr Pro Leu Pro Val Ile Gly Asn Ile Leu Gln Ile Gly Ile Lys
 35 40 45
 Asp Ile Ser Lys Ser Leu Thr Asn Leu Ser Lys Val Tyr Gly Pro Val
 50 55 60
 Phe Thr Leu Tyr Phe Gly Leu Lys Pro Ile Val Val Leu His Gly Tyr
 65 70 75 80
 Glu Ala Val Lys Glu Ala Leu Ile Asp Leu Gly Glu Glu Phe Ser Gly
 85 90 95
 Arg Gly Ile Phe Pro Leu Ala Glu Arg Ala Asn Arg Gly Phe Gly Ile
 100 105 110
 Val Phe Ser Asn Gly Lys Lys Trp Lys Glu Ile Arg Arg Phe Ser Leu
 115 120 125
 Met Thr Leu Arg Asn Phe Gly Met Gly Lys Arg Ser Ile Glu Asp Arg
 130 135 140
 Val Gln Glu Glu Ala Arg Cys Leu Val Glu Glu Leu Arg Lys Thr Lys
 145 150 155 160
 Ala Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn
 165 170 175
 Val Ile Cys Ser Ile Ile Phe His Lys Arg Phe Asp Tyr Lys Asp Gln
 180 185 190
 Gln Phe Leu Asn Leu Met Glu Lys Leu Asn Glu Asn Ile Lys Ile Leu
 195 200 205

```

Ser Ser Pro Trp Ile Gln Ile Cys Asn Asn Phe Ser Pro Ile Ile Asp
210                215                220

Tyr Phe Pro Gly Thr His Asn Lys Leu Leu Lys Asn Val Ala Phe Met
225                230                235                240

Lys Ser Tyr Ile Leu Glu Lys Val Lys Glu His Gln Glu Ser Met Asp
                245                250                255

Met Asn Asn Pro Gln Asp Phe Ile Asp Cys Phe Leu Met Lys Met Glu
                260                265                270

Lys Glu Lys His Asn Gln Pro Ser Glu Phe Thr Ile Glu Ser Leu Glu
                275                280                285

Asn Thr Ala Val Asp Leu Phe Gly Ala Gly Thr Glu Thr Thr Ser Thr
290                295                300

Thr Leu Arg Tyr Ala Leu Leu Leu Leu Leu Lys His Pro Glu Val Thr
305                310                315                320

Ala Lys Val Gln Glu Glu Ile Glu Arg Val Ile Gly Arg Asn Arg Ser
                325                330                335

Pro Cys Met Gln Asp Arg Ser His Met Pro Tyr Thr Asp Ala Val Val
                340                345                350

His Glu Val Gln Arg Tyr Ile Asp Leu Leu Pro Thr Ser Leu Pro His
                355                360                365

Ala Val Thr Cys Asp Ile Lys Phe Arg Asn Tyr Leu Ile Pro Lys Gly
370                375                380

Thr Thr Ile Leu Ile Ser Leu Thr Ser Val Leu His Asp Asn Lys Glu
385                390                395                400

Phe Pro Asn Pro Glu Met Phe Asp Pro His His Phe Leu Asp Glu Gly
                405                410                415

Gly Asn Phe Lys Lys Ser Lys Tyr Phe Met Pro Phe Ser Ala Gly Lys
                420                425                430

Arg Ile Cys Val Gly Glu Ala Leu Ala Gly Met Glu Leu Phe Leu Phe
                435                440                445

Leu Thr Ser Ile Leu Gln Asn Phe Asn Leu Lys Ser Leu Val Asp Pro
450                455                460

Lys Asn Leu Asp Thr Thr Pro Val Val Asn Gly Phe Ala Ser Val Pro
465                470                475                480

Pro Phe Tyr Gln Leu Cys Phe Ile Pro Val
                485                490

```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1852 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GAAGGCTTCA ATGGATTCTC TTGTGGTCCT TGTCCTCTGT CTCTCATGTT TGCTTCTCCT	60
TTCACCTCTGG AGACAGAGCT CTGGGAGAGG AAAACTCCCT CCTGGCCCCA CTCCTCTCCC	120
AGTGATTGGA AATATCCTAC AGATAGGTAT TAAGGACATC AGCAAATCCT TAACCAATCT	180
CTCAAAGGTC TATGGCCCTG TGTTCACCTCT GTATTTTGGC CTGAAACCCA TAGTGGTGCT	240
GCATGGATAT GAAGCAGTGA AGGAAGCCCT GATTGATCTT GGAGAGGAGT TTTCTGGAAG	300
AGGCATTTTC CCACTGGCTG AAAGAGCTAA CAGAGGATTT GGAATTGTTT TCAGCAATGG	360
AAAGAAATGG AAGGAGATCC GGCCTTTCTC CCTCATGACG CTGCGGAATT TTGGGATGGG	420
GAAGAGGAGC ATTGAGGACC GTGTTCAAGA GGAAGCCCGC TGCCTTGTGG AGGAGTTGAG	480
AAAAACCAAG GCCTCACCTT GTGATCCAC TTTTCATCTG GGCTGTGCTC CCTGCAATGT	540
GATCTGCTCC ATTATTTTCC ATAAACGTTT TGATTATAAA GATCAGCAAT TTCTTAACTT	600
AATGGAAAAG TTGAATGAAA ACATCAAGAT TTTGAGCAGC CCCTGGATCC AGATCTGCAA	660
TAATTTTTCT CCTATCATTG ATTACTTCCC GGGAACTCAC AACAAATTAC TTA AAAACGT	720
TGCTTTTATG AAAAGTTATA TTTTGGAAAA AGTAAAAGAA CACCAAGAAT CAATGGACAT	780
GAACAACCTT CAGGACTTTA TTGATTGCTT CCTGATGAAA ATGGAGAAGG AAAAGCACAA	840
CCAACCATCT GAATTTACTA TTGAAAGCTT GGAAAACACT GCAGTTGACT TGT TTGGAGC	900
TGGGACAGAG ACGACAAGCA CAACCCTGAG ATATGCTCTC CTTCTCCTGC TGAAGCACCC	960
AGAGGTCACA GCTAAAGTCC AGGAAGAGAT TGAACGTGTG ATTGGCAGAA ACCGGAGCCC	1020
CTGCATGCAA GACAGGAGCC ACATGCCCTA CACAGATGCT GTGGTGACG AGGTCCAGAG	1080
ATACATTGAC CTTCTCCCCA CCAGCCTGCC CCATGCAGTG ACCTGTGACA TTAAATTCAG	1140
AACTATCTC ATTCCCAAGG GCACAACCAT ATTAATTTCC CTGACTTCTG TGCTACATGA	1200
CAACAAAGAA TTTCCCAACC CAGAGATGTT TGACCCTCAT CACTTTCTGG ATGAAGGTGG	1260
CAATTTTAAG AAAAGTAAAT ACTTCATGCC TTTCTCAGCA GGAAAACGGA TTTGTGTGGG	1320
AGAAGCCCTG GCCGGCATGG AGCTGTTTTT ATTCCTGACC TCCATTTTAC AGA ACTTTAA	1380
CCTGAAATCT CTGGTTGACC CAAAGAACCT TGACACCACT CCAGTTGTCA ATGGATTGTC	1440
CTCTGTGCCG CCCTTCTACC AGCTGTGCTT CATTCCTGTC TGAAGAAGAG CAGATGGCCT	1500
GGCTGCTGCT GTGCAGTCCC TGCAGCTCTC TTTCTCTGG GCATTATCC ATCTTTCACT	1560
ATCTGTAATG CCTTTTCTCA CTGTCTATCT CACATTTTCC CTTCCTGAA GATCTAGTGA	1620
ACATTGACC TCCATTACGG AGAGTTTCCT ATGTTTCACT GTGCAAATAT ATCTGCTATT	1680
CTCCATACTC TGTAACAGTT GCATTGACTG TCACATAATG CTCATACTTA TCTAATGTTG	1740
AGTTATTAAT ATGTTATTAT TAAATAGAGA AATATGATTT GTGTATTATA ATTCAAAGGC	1800
ATTTCTTTTC TGCATGTTCT AAATAAAAAG CATTATTATT TGCTGAAAAA AA	1852

105

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 490 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Met Asp Pro Ala Val Ala Leu Val Leu Cys Leu Ser Cys Leu Phe Leu
 1           5           10           15
Leu Ser Leu Trp Arg Gln Ser Ser Gly Arg Gly Arg Leu Pro Ser Gly
 20           25           30
Pro Thr Pro Leu Pro Ile Ile Gly Asn Ile Leu Gln Leu Asp Val Lys
 35           40           45
Asp Met Ser Lys Ser Leu Thr Asn Phe Ser Lys Val Tyr Gly Pro Val
 50           55           60
Phe Thr Val Tyr Phe Gly Leu Lys Pro Ile Val Val Leu His Gly Tyr
 65           70           75           80
Glu Ala Val Lys Glu Ala Leu Ile Asp His Gly Glu Glu Phe Ser Gly
 85           90           95
Arg Gly Ser Phe Pro Val Ala Glu Lys Val Asn Lys Gly Leu Gly Ile
 100          105          110
Leu Phe Ser Asn Gly Lys Arg Trp Lys Glu Ile Arg Arg Phe Cys Leu
 115          120          125
Met Thr Leu Arg Asn Phe Gly Met Gly Lys Arg Ser Ile Glu Asp Arg
 130          135          140
Val Gln Glu Glu Ala Arg Cys Leu Val Glu Glu Leu Arg Lys Thr Asn
 145          150          155          160
Ala Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn
 165          170          175
Val Ile Cys Ser Val Ile Phe His Asp Arg Phe Asp Tyr Lys Asp Gln
 180          185          190
Arg Phe Leu Asn Leu Met Glu Lys Phe Asn Glu Asn Leu Arg Ile Leu
 195          200          205
Ser Ser Pro Trp Ile Gln Val Cys Asn Asn Phe Pro Ala Leu Ile Asp
 210          215          220
Tyr Leu Pro Gly Ser His Asn Lys Ile Ala Glu Asn Phe Ala Tyr Ile
 225          230          235          240
Lys Ser Tyr Val Leu Glu Arg Ile Lys Glu His Gln Glu Ser Leu Asp
 245          250          255
Met Asn Ser Ala Arg Asp Phe Ile Asp Cys Phe Leu Ile Lys Met Glu
 260          265          270
Gln Glu Lys His Asn Gln Gln Ser Glu Phe Thr Val Glu Ser Leu Ile
 275          280          285

```

106

Ala Thr Val Thr Asp Met Phe Gly Ala Gly Thr Glu Thr Thr Ser Thr
 290 295 300

Thr Leu Arg Tyr Gly Leu Leu Leu Leu Lys Tyr Pro Glu Val Thr
 305 310 315 320

Ala Lys Val Gln Glu Glu Ile Glu Cys Val Val Gly Arg Asn Arg Ser
 325 330 335

Pro Cys Met Gln Asp Arg Ser His Met Pro Tyr Thr Asp Ala Val Val
 340 345 350

His Glu Ile Gln Arg Tyr Ile Asp Leu Leu Pro Thr Asn Leu Pro His
 355 360 365

Ala Val Thr Cys Asp Val Lys Phe Lys Asn Tyr Leu Ile Pro Lys Gly
 370 375 380

Met Thr Ile Ile Thr Ser Leu Thr Ser Val Leu His Asn Asp Lys Glu
 385 390 395 400

Phe Pro Asn Pro Glu Met Phe Asp Pro Gly His Phe Leu Asp Lys Ser
 405 410 415

Gly Asn Phe Lys Lys Ser Asp Tyr Phe Met Pro Phe Ser Ala Gly Lys
 420 425 430

Arg Met Cys Met Gly Glu Gly Leu Ala Arg Met Glu Leu Phe Leu Phe
 435 440 445

Leu Thr Thr Ile Leu Gln Asn Phe Asn Leu Lys Ser Gln Val Asp Pro
 450 455 460

Lys Asp Ile Asp Ile Thr Pro Ile Ala Asn Ala Phe Gly Arg Val Pro
 465 470 475 480

Pro Leu Tyr Gln Leu Cys Phe Ile Pro Val
 485 490

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2258 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGTGAAAGCC CGCAGTTGTC TTAATAAGAA GAGAAGCCTT CAATGGATCC AGCTGTGGCT	60
CTGGTGCTCT GTCTCTCCTG TTTGTTTCTC CTTTCACTCT GGAGGCAGAG CTCTGGAAGA	120
GGGAGGCTCC CGTCTGGCCC CACTCCTCTC CCGATTATTG GAAATATCCT GCAGTTAGAT	180
GTAAAGGACA TGAGCAAATC CTTAACCAAT TTCTCAAAG TCTATGGCCC TGTGTTCACT	240
GTGTATTTTG GCCTGAAGCC CATTGTGGTG TTGCATGGAT ATGAAGCAGT GAAGGAGGCC	300
CTGATTGATC ATGGAGAGGA GTTTTCTGGA AGAGGAAGTT TTCCAGTGGC TGAAAAAGTT	360
AACAAAGGAC TTGGAATCCT TTTCAGCAAT GGAAAGAGAT GGAAGGAGAT CCGGCGTTTC	420
TGCCTCATGA CTCTGCGGAA TTTTGGGATG GGGAAGAGGA GCATCGAGGA CCGTGTTCAA	480

GAGGAAGCCC GCTGCCTTGT GGAGGAGTTG AGAAAAACCA ATGCCTCACC CTGTGATCCC	540
ACTTTCATCC TGGGCTGTGC TCCCTGCAAT GTGATCTGCT CTGTTATTTT CCATGATCGA	600
TTTGATTATA AAGATCAGAG GTTCTTAAC TTGATGGAAA AATTCAATGA AAACCTCAGG	660
ATTCTGAGCT CTCCATGGAT CCAGGTCTGC AATAATTTCC CTGCTCTCAT CGATTATCTC	720
CCAGGAAGTC ATAATAAAT AGCTGAAAAT TTTGCTTACA TTAAAAGTTA TGTATTGGAG	780
AGAATAAAAG AACATCAAGA ATCCCTGGAC ATGAACAGTG CTCGGGACTT TATTGATTGT	840
TTCCTGATCA AAATGGAACA GGAAAAGCAC AATCAACAGT CTGAATTTAC TGTTGAAAGC	900
TTGATAGCCA CTGTAAGTGA TATGTTTGGG GCTGGAACAG AGACAACGAG CACCACTCTG	960
AGATATGGAC TCCTGCTCCT GCTGAAGTAC CCAGAGGTCA CAGCTAAAGT CCAGGAAGAG	1020
ATTGAATGTG TAGTTGGCAG AAACCGGAGC CCCTGTATGC AGGACAGGAG TCACATGCCC	1080
TACACAGATG CTGTGGTGCA CGAGATCCAG AGATACATTG ACCTCCTCCC CACCAACCTG	1140
CCCCATGCAG TGACCTGTGA TGTTAAATTC AAAAATACC TCATCCCCAA GGGCATGACC	1200
ATAATAACAT CCCTGACTTC TGTGCTGCAC AATGACAAAG AATTCCCCAA CCCAGAGATG	1260
TTTGACCCTG GCCACTTTCT GGATAAGAGT GGCAACTTTA AGAAAAGTGA CTACTTCATG	1320
CCTTCTCAG CAGGAAAACG GATGTGTATG GGAGAGGGCC TGGCCCGCAT GGAGCTGTTT	1380
TTATTCTGA CCACCATTTT GCAGAACTTT AACCTGAAAT CTCAGGTTGA CCCAAAGGAT	1440
ATTGACATCA CCCCCATTGC CAATGCATTT GGTGCTGTGC CACCTTGTGA CCAGCTCTGC	1500
TTCAATCCTG TCTGAAGAAG GGCAGATAGT TTGGCTGCTC CTGTGCTGTC ACCTGCAATT	1560
CTCCCTTATC AGGGCCATTG GCCTCTCCCT TCTCTCTATG AGGGATATTT TCTCTGACTT	1620
GTCAATCCAC ATCTTCCCAT TCCCTCAAGA TCCAATGAAC ATCCAACCTC CATTAAAGAG	1680
AGTTTCTTGG GTCACCTCCT AAATATATCT GCTATTCTCC ATACTCTGTA TCACCTGTAT	1740
TGACCACCAC ATATGCTAAT ACCTATCTAC TGCTGAGTTG TCAGTATGTT ATCACTATAA	1800
AACAAAGAAA AATGATTAAAT AAATGACAAT TCAGAGCCAT TTATTCTCTG CATGCTCTAG	1860
ATAAAAATGA TTATTATTTA CTGGGTCACT TCTTAGATTT CTTCCTTTTG AGTAAAATGA	1920
AAGTAAGAAA TGAAAGAAAA TAGAATGTGA AGAGGCTGTG CTGGCCCTCA TAGTGTTAAG	1980
CACAAAAGG GAGAAAGGTA AGAGGGTAGG AAAGCTGTTT TAGCTAAATG CCACCTAGAG	2040
TTATTGGAGG TCTGAATTTG GAAAAAATA CTATGTCCAG GAGCAGCTGT AACCTGTAGG	2100
GAAATAATGG AACATCATC CATAAGAGGG ATGAACATTA AGTGTTTGAA TTCATGCTCT	2160
GCTTTTGTGT TACTGTAAAC ACAAGATCAA GATTTGGATA ATCTTTTCC TTTGTGTTTC	2220
CAACTTAGAT CATGTCTAAA TATATGCTTT CATATGGC	2258

(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 490 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

Met Asp Pro Xaa Val Val Leu Val Leu Cys Leu Ser Cys Leu Leu Leu
1          5          10          15

Leu Ser Leu Trp Arg Gln Ser Ser Gly Arg Gly Lys Leu Pro Pro Gly
20          25          30

Pro Thr Pro Leu Pro Xaa Ile Gly Asn Ile Leu Gln Ile Asp Xaa Lys
35          40          45

Asp Ile Ser Lys Ser Leu Thr Asn Xaa Ser Lys Val Tyr Gly Pro Val
50          55          60

Phe Thr Xaa Tyr Phe Gly Leu Lys Pro Ile Val Val Leu His Gly Tyr
65          70          75          80

Glu Ala Val Lys Glu Ala Leu Ile Asp Leu Gly Glu Glu Phe Ser Gly
85          90          95

Arg Gly Xaa Phe Pro Leu Ala Glu Arg Ala Asn Xaa Gly Xaa Gly Ile
100         105         110

Val Phe Ser Asn Gly Lys Arg Trp Lys Glu Ile Arg Arg Phe Ser Leu
115         120         125

Met Thr Leu Arg Asn Phe Gly Met Gly Lys Arg Ser Ile Glu Asp Arg
130         135         140

Val Gln Glu Glu Ala Arg Cys Leu Val Glu Glu Leu Arg Lys Thr Lys
145         150         155         160

Ala Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn
165         170         175

Val Ile Cys Ser Xaa Ile Phe His Lys Arg Phe Asp Tyr Lys Asp Gln
180         185         190

Gln Phe Leu Asn Leu Met Glu Lys Xaa Asn Glu Asn Ile Arg Ile Leu
195         200         205

Ser Ser Pro Trp Ile Gln Xaa Cys Asn Asn Phe Pro Xaa Xaa Ile Asp
210         215         220

Tyr Phe Pro Gly Thr His Asn Lys Leu Leu Lys Asn Val Ala Phe Met
225         230         235         240

Lys Ser Tyr Ile Leu Glu Lys Val Lys Glu His Gln Glu Ser Xaa Asp
245         250         255

Met Asn Asn Pro Arg Asp Phe Ile Asp Cys Phe Leu Ile Lys Met Glu
260         265         270

Xaa Glu Lys His Asn Gln Gln Ser Glu Phe Thr Ile Glu Ser Leu Xaa
275         280         285

```

109

Xaa Thr Xaa Xaa Asp Leu Phe Gly Ala Gly Thr Glu Thr Thr Ser Thr
 290 295 300
 Thr Leu Arg Tyr Xaa Leu Leu Leu Leu Lys His Pro Glu Val Thr
 305 310 315 320
 Ala Lys Val Gln Glu Glu Ile Glu Arg Val Ile Gly Arg Asn Arg Ser
 325 330 335
 Pro Cys Met Gln Asp Arg Ser His Met Pro Tyr Thr Asp Ala Val Val
 340 345 350
 His Glu Xaa Gln Arg Tyr Ile Asp Leu Leu Pro Thr Ser Leu Pro His
 355 360 365
 Ala Val Thr Cys Asp Val Lys Phe Arg Asn Tyr Leu Ile Pro Lys Gly
 370 375 380
 Thr Thr Ile Leu Thr Ser Leu Thr Ser Val Leu His Asp Xaa Lys Glu
 385 390 395 400
 Phe Pro Asn Pro Glu Met Phe Asp Pro Gly His Phe Leu Asp Xaa Gly
 405 410 415
 Gly Asn Phe Lys Lys Ser Asp Tyr Phe Met Pro Phe Ser Ala Gly Lys
 420 425 430
 Arg Ile Cys Val Gly Glu Gly Leu Ala Arg Met Glu Leu Phe Leu Phe
 435 440 445
 Leu Thr Thr Ile Leu Gln Asn Phe Asn Leu Lys Ser Leu Val Asp Pro
 450 455 460
 Lys Xaa Leu Asp Thr Thr Pro Val Val Asn Gly Phe Ala Ser Val Pro
 465 470 475 480
 Pro Phe Tyr Gln Leu Cys Phe Ile Pro Val
 485 490

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1892 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGTGAAAGCC CGCAGTTGTC TTAAGAGAA GAGAAGNCTT CAATGGATCC TTTTGTGGTC 60
 CTNGTGCTCT GTCTCTCATG TTTGCTTCTC CTTTCACTCT GGAGACAGAG CTCTGGGAGA 120
 GGNAACTCC CTCCTGGCCC CACTCCTCTC CCANTNATTG GAAATATCCT ACAGATAGAT 180
 NTTAAGGACA TCAGCAAATC CTTAACCAAT NTCTCAAAAG TCTATGGCCC TGTGTTCACT 240
 NTGTATTTTG GCCTGAAACC CATAGTGGTG NTGCATGGAT ATGAAGCAGT GAAGGAAGCC 300
 CTGATTGATC NTGGAGAGGA GTTTTCTGGA AGAGGCANTT TCCCACTGGC TGAAAGAGNT 360
 AACANAGGAN TTGGAATCGT TTTGAGCAAT GGAAAGAGAT GGAAGGAGAT CCGGCGTTTC 420

110

```

TCCCTCATGA CGCTGCGGAA TTTTGGGATG GGAAGAGGA GCATTGAGGA CCGTGTTCAA      480
GAGGAAGCCC GCTGCCTTGT GGAGGAGTTG AGAAAAACCA AGGCCTCACC CTGTGATCCC      540
ACTTTCATCC TGGGCTGTGC TCCCTGCAAT GTGATCTGCT CCNTTATTTT CCATAAACGN      600
TTTGATTATA AAGATCAGNA ATTTCTTAAC TTGATGGAAA AATTNAATGA AAACATCAGG      660
ATTCTGAGCN CCCCNTGGAT CCAGNTCTGC AATAATTTCN CTCCTNTCAT TGATTATTTT      720
CCNGGAAGTC ANAACAAATT ACTTAAAAAN GTTGCTTTTA TGAAAAGTTA TATTTTGGAG      780
AAAGTAAAAG AACACCAAGA ATCANTGGAC ATGAACAANC CTCGGGACTT TATTGATTGC      840
TTCTGATCA AAATGGAGNA GGAAAAGCAC AACCAACAGT CTGAATTTAC TATTGAAAGC      900
TTGGTANNCA CTGNAGCTGA NTTGTTTGA GCTGGNACAG AGACAACAAG CACNACNCTG      960
AGATATGNNC TCCTNCTCCT GCTGAAGCAC CCAGAGGTCA CAGCTAAAGT CCAGGAAGAG     1020
ATTGAACGTG TAATTGGCAG AAACCGGAGC CCCTGCATGC AGGACAGGAG CCACATGCCC     1080
TACACAGATG CTGTGGTGCA CGAGNTCCAG AGATACATTG ACCTNCTCCC CACCAGCCTG     1140
CCCCATGCAG TGACCTGTGA NNTTAAATTC AGAACTACC TCATNCCCAA GGGCACAACC     1200
ATANTAACNT CCCTGACTTC TGTGCTACAT GANNACAAAG AATTTCCCAA CCCAGAGATG     1260
TTTGACCTN GNCACTTTCT GGATNANNGT GGCAANTTTA AGAAAAGTNA CTACTTCATG     1320
CCTTCTCAG CAGGAAAACG GATTTGTGTG GGAGANGGCC TGGCCCGCAT GGAGCTGTTT     1380
TTATTCCTGA CCNCCATTTT ACAGAACTTT AACCTGAAAT CTCTGGTTGA CCCAAANGAC     1440
CTTGACACCA CTCCAGTTGN CAATGGATTT GCTTCTGTGC CNCCCTTCTA CCAGCTNTGC     1500
TTCATTCTCG TCTGAAGAAG GGCAGATGGT CTGGCTGCTN CTGTGCTGTC NNNNNNTN     1560
NNTTNTNTCT GGGGCAATTT CCNTCTTNC TNNNTNTTN TGCNNTTNT CATCTGNCAT     1620
CTCACANTNC NNCTTCCCTT ANCATCNAGN NACCATTNAN NNNCAATNTC CAAGAGNGTG     1680
NNTTNTTN CTNTCCACCT ANATCTATCN NTNNNNCTNC TNTNTNTNNA TNACTTTGAT     1740
TGTCNCTAN TGATGNTAAT TTTTAATAT TGNNTTATTG NNANNTNTT ATNANTNANA     1800
AANAATGAT AATTNTNTN AAATNNNAAG TCANTGCNNT TNANNATNTN CNNAATAAAA     1860
AGCATTATTA TTTGCTGAAA AAAAGTCAGT TC                                     1892

```

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCAAGCTTAA AAAATGGATC CAGCTGTGGC TCT

33

111

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GCAAGCTTGC CAAACTATCT GCCCTTCT

28

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ACTTTTCAAT GTAAGCAAAT

20

(2) INFORMATION FOR SEQ ID NO:18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTAGTAATTC TTTGAGATAT

20

(2) INFORMATION FOR SEQ ID NO:19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTGTTAGCTC TTTCAGCCAG

20

112

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGAGCACAGC CCAGGATGAA

20

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCAAGCTTAA AAAATGGATC CAGCTGTGGC TCT

33

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCAAGCTTGC CAAACTATCT GCCCTTCT

28

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TGGCCCTGAT AAGGGAGAAT

20

113

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

ATCCAGAGAT ACATTGACCT C

21

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

CCATGAAGTG ACCTGTGATG

20

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AAAGATGGAT AATGCCCCAG

20

(2) INFORMATION FOR SEQ ID NO:27:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GAAGGAGATC CGGCGTTTCT

20

114

(2) INFORMATION FOR SEQ ID NO:28:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GGCGTTTCTC CCTCATGACG

20

(2) INFORMATION FOR SEQ ID NO:29:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TTGTCATTGT GCAG

14

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CACATGCCCT ACACA

15

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

TGACGCTGCG GAATT

15

115

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 15 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GGACTTTATT GATTG

15

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 19 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

ATGATTCTCT TGTGGTCCT

19

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

AAAGATGGAT AATGCCCCCA G

21

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCAAGCTTAA AAAAATGGAA CCTTTTGTGG TCCT

34

116

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GCAAGCTTGC CAGATGGGCT AGCATTCT

28

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCAAGCTTAA AAAAATGGAT TCTCTTGTGG TCCT

34

(2) INFORMATION FOR SEQ ID NO:38:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GCAAGCTTGC CAGGCCATCT GCTCTTCT

28

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GCAAGCTTAA AAAAATGGAT TCTCTTGTGG TCCT

34

117

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GCAAGCTTGC CAGACCATCT GTGCTTCT

28

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (oligo)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

AGCTTAAAAA AATG

14

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 14 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: DNA (oligo)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

GATCCATTTT TTTA

14

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: unknown
 - (D) TOPOLOGY: unknown

- (ii) MOLECULE TYPE: peptide

118

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Cys Ile Asp Tyr Leu Pro Gly Ser His Asn Lys Ile Ala Glu Asn Phe
 1 5 10 15
 Ala

(2) INFORMATION FOR SEQ ID NO:44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: unknown
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

Cys Leu Ala Phe Met Glu Ser Asp Ile Leu Glu Lys Val Lys
 1 5 10

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 284 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..283

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

A TTG AAT GAA AAC ATC AGG ATT GTA AGC ACC CCC TGG ATC CAG ATA	46
Leu Asn Glu Asn Ile Arg Ile Val Ser Thr Pro Trp Ile Gln Ile	
1 5 10 15	
TGC AAT AAT TTT CCC ACT ATC ATT GAT TAT TTC CCG GGA ACC CAT AAC	94
Cys Asn Asn Phe Pro Thr Ile Ile Asp Tyr Phe Pro Gly Thr His Asn	
20 25 30	
AAA TTA CTT AAA AAC CTT GCT TTT ATG GAA AGT GAT ATT TTG GAG AAA	142
Lys Leu Leu Lys Asn Leu Ala Phe Met Glu Ser Asp Ile Leu Glu Lys	
35 40 45	
GTA AAA GAA CAC CAA GAA TCG ATG GAC ATC AAC AAC CCT CGG GAC TTT	190
Val Lys Glu His Gln Glu Ser Met Asp Ile Asn Asn Pro Arg Asp Phe	
50 55 60	
ATT GAT TGC TTC CTG ATC AAA ATG GAG AAG GAA AAG CAA AAC CAA CAG	238
Ile Asp Cys Phe Leu Ile Lys Met Glu Lys Glu Lys Gln Asn Gln Gln	
65 70 75	
TCT GAA TTC ACT ATT GAA AAC TTG GTA ATC ACT GCA GCT GAC TTA	283
Ser Glu Phe Thr Ile Glu Asn Leu Val Ile Thr Ala Ala Asp Leu	
80 85 90	

C

284

119

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 94 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

```

Leu Asn Glu Asn Ile Arg Ile Val Ser Thr Pro Trp Ile Gln Ile Cys
 1             5             10             15
Asn Asn Phe Pro Thr Ile Ile Asp Tyr Phe Pro Gly Thr His Asn Lys
          20             25             30
Leu Leu Lys Asn Leu Ala Phe Met Glu Ser Asp Ile Leu Glu Lys Val
          35             40             45
Lys Glu His Gln Glu Ser Met Asp Ile Asn Asn Pro Arg Asp Phe Ile
          50             55             60
Asp Cys Phe Leu Ile Lys Met Glu Lys Glu Lys Gln Asn Gln Gln Ser
          65             70             75             80
Glu Phe Thr Ile Glu Asn Leu Val Ile Thr Ala Ala Asp Leu
          85             90

```

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 244 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 44..103

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

```

ATTGAATGAA AACATCAGGA TTGTAAGCAC CCCCTGGATC CAG GAA CCC ATA ACA      55
                                     Glu Pro Ile Thr
                                     1
AAT TAC TTA AAA ACC TTG CTT TTA TGG AAA GTG ATA TTT TGG AGA AAG      103
Asn Tyr Leu Lys Thr Leu Leu Leu Trp Lys Val Ile Phe Trp Arg Lys
 5             10             15             20
TAAAGAACA CCAAGAATCG ATGGACATCA ACAACCCTCG GGACTTTATT GATTGCTTCC      163
TGATCAAAAT GGAGAAGGAA AAGCAAAACC AACAGTCTGA ATTCACTATT GAAAACTTGG      223
TAATCACTGC AGCTGACTTA C                                             244

```

120

(2) INFORMATION FOR SEQ ID NO:48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

Glu Pro Ile Thr Asn Tyr Leu Lys Thr Leu Leu Leu Trp Lys Val Ile
1 5 10 15
Phe Trp Arg Lys
20

(2) INFORMATION FOR SEQ ID NO:49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 83 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1..32

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 33..83

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

TTTTAATTTA ATAAATTATT GTTTTCTCTT AGATATGCAA TAATTTTCCC ACTATCAT TG 60
ATTATTTCCC GGGAACCCAT AAC 83

(2) INFORMATION FOR SEQ ID NO:50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 83 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: intron
- (B) LOCATION: 1..72

(ix) FEATURE:

- (A) NAME/KEY: exon
- (B) LOCATION: 73..83

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

TTTTAATTTA ATAAATTATT GTTTTCTCTT AGATATGCAA TAATTTTCCC ACTATCAT TG 60
ATTATTTCCA AGGAACCCAT AAC 83

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 826 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

```

ATGGTGATGT AGNAANTCAT NCCATCTTAT ATTTCNAGAG TGTAGAGGAG GATTGTTGNG      60
GAAGTAAGAG GNNTAAGATA GAGATGCNTT TATACTATCC CAAGCAGGGA TRAGTCTAGG      120
AAATGATTAT CGTCTTTGAT TCTCTTGTC AGRATTTCTT TCTCMNATCT TGTATAATCA      180
GAGAATTACT ACACATGGAC AATRAARATT TCCCCNTCCA GATANACAAT ATATTTTATT      240
TATATTTATA GTTTTAAATT ACAACCAGAG CTTGGCATAT TGTATCTATA CCTTTAATAA      300
ATGCTTTTAA TTTAATAAAT TATTGTTTTT TCTTAGATAT GCAATAATTT TCCCACTATC      360
ATTGATTATT TCCCGGGAAC CCATAACAAA TTAAGTAAAA ACCTTGCTTT TATGGAAAGT      420
GATATTTTGG AGAAAGTAAA AGAACACCAA GAATCGATGG ACATCAACAA CCCTCGGGAC      480
TTTATTGATT GCTTCCTGAT CAAAATGGAG AAGGTAAAAA GTTAACAAAA GCTTAGTTAT      540
GTGACTGCTT GCGTATKTGT GATTCATTGA CTAGTTGKGT GTTTACTACG GATGTTTAAAC      600
AGGTCAAGGA GTAATGCTTG AGAAGCATAT TTAAGTTTTT ATTGTATGCA TGAATATCCA      660
GTAAGCATCA TAGAAAATGT AAAATTAANT TGTTAAATAA TTAGAATACA TAGAAGAAAT      720
TGTTTAGATA AATATNATCT ATCTGAACAA TAAGGATGTC AGGATAGGAA AAGCTCTGTT      780
TCTGCAGCTT CCAAGTGGAGA TCAGCACAGG AGGGAACTTA TTTTTT      826

```

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 655 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 263..421

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

```

AGGGAAAAGA CAAATAGGCC GGGGATGNAA ATTTAGCATG TGAGCAACCT TANTTAACCA      60
GCTAGGCTGT AATTGNTAAT TCGAGANTAA TGTNAAAGTG ATGTGTTGAT TTTATGCATG      120
CCNNACTCNT TTTTGCTTTT AAGGGGAGTC ATAGGTAAGA TATTACTTAA AATTTCTAAA      180

```

122

CTATTATTAT	CTGTAACTA	ATATGAAGTG	TTTTATATCT	AATGTTTACT	CATATTTTAA	240
AATTGTTTTCC	AATCATTTAG	CT TCA CCC	TGT GAT CCC	ACT TTC ATC	CTG GGC	292
		Ser Pro Cys	Asp Pro Thr	Phe Ile Leu	Gly 10	
		1		5		
TGT GCT CCC	TGC AAT GTG	ATC TGC TCC	ATT ATT TTC	CAG AAA CGT	TTC	340
Cys Ala Pro	Cys Asn Val	Ile Cys Ser	Ile Ile Phe	Gln Lys Arg	Phe 25	
	15		20			
GAT TAT AAA	GAT CAG CAA	TTT CTT AAC	TTG ATG GAA	AAA TTG AAT	GAA	388
Asp Tyr Lys	Asp Gln Gln	Phe Leu Asn	Leu Met Glu	Lys Leu Asn	Glu 40	
	30		35			
AAC ATC AGG	ATT GTA AGC	ACC CCC TGG	ATC CAG GTAAGGACA	AGTTTTGTGC		440
Asn Ile Arg	Ile Val Ser	Thr Pro Trp	Ile Gln			
	45		50			
TTCCTGAGAA	ACCACTTACA	GTCTTTTTTT	CTGGGAAATC	CAAAATTCTA	TATTGACCAA	500
GCCCTGAAGT	ACATTTGTGA	ATACTACAGT	CTTGCCTAGA	CAGCCATGGG	GTGAATATCT	560
GGAAAAGATG	GCAAAGNTCT	TTATTTTATG	CACAGGAAAT	GAATATCCCA	ATATAGATCA	620
GGCTTCTAAG	CCCATTAGCT	CCCTGATCAG	TGTTT			655

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 53 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

Ser Pro Cys Asp Pro Thr Phe Ile Leu Gly Cys Ala Pro Cys Asn Val
1 5 10 15
Ile Cys Ser Ile Ile Phe Gln Lys Arg Phe Asp Tyr Lys Asp Gln Gln
20 25 30
Phe Leu Asn Leu Met Glu Lys Leu Asn Glu Asn Ile Arg Ile Val Ser
35 40 45
Thr Pro Trp Ile Gln
50

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 292 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

ATGAAGTGTT TTATATCTAA TGTTTACTCA TATTTTAAAA TTGTTTCCAA TCATTTAGCT 60
TCACCCCTGTG ATCCCACTTT CATCCTGGGC TGTGCTCCCT GCAATGTGAT CTGCTCCATT 120

123

ATTTTCCAGA AACGTTTCGA TTATAAAGAT CAGCAATTTC TTAAGTTGAT GGAAAAATTG 180
AATGAAAACA TCAGGATTGT AAGCACCCCC TGAATCCAGG TAAGGACAAG TTTTGTGCTT 240
CCTGAGAAAC CACTTACAGT CTTTTTTTCT GGGAAATCCA AAATTCTATA TT 292

(2) INFORMATION FOR SEQ ID NO:55:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

AATTACAACC AGAGCTTGGC 20

(2) INFORMATION FOR SEQ ID NO:56:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 22 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

TATCACTTTC CATAAAAGCA AG 22

(2) INFORMATION FOR SEQ ID NO:57:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

TATTATCTGT TAACTAACTA ATATGA 26

(2) INFORMATION FOR SEQ ID NO:58:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

124

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

ACTTCAGGGC TTGGTCAATA

20

(2) INFORMATION FOR SEQ ID NO:59:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

ATTGAATGAA AACATCAGGA TTG

23

(2) INFORMATION FOR SEQ ID NO:60:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (primer)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

GTAAGTCAGC TGCAGTGATT A

21

(2) INFORMATION FOR SEQ ID NO:61:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 826 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

ATGGTGATGT AGNAANTCAT NCCATCTTAT ATTCNAGAG TGTAGAGGAG GATTGTTGNG	60
GAAGTAAGAG GNNTAAGATA GAGATGCNTT TATACTATCC CAAGCAGGGA TRAGTCTAGG	120
AAATGATTAT CGTCTTTGAT TCTCTTGTC GRATTTTCTT TCTCMNATCT TGTATAATCA	180
GAGAATTACT ACACATGGAC AATRAARATT TCCCCNTCCA GATANACAAT ATATTTTATT	240
TATATTTATA GTTTTAAATT ACAACCAGAG CTTGGCATAT TGTATCTATA CCTTTAATAA	300
ATGCTTTTAA TTTAATAAAT TATTGTTTTT TCTTAGATAT GCAATAATTT TCCCACTATC	360
ATTGATTATT TCCCAGGAAC CCATAACAAA TTACTTAAAA ACCTTGCTTT TATGGAAAGT	420
GATATTTTGG AGAAAGTAAA AGAACACCAA GAATCGATGG ACATCAACAA CCCTCGGGAC	480

125

TTTATTGATT GCTTCCTGAT CAAAATGGAG AAGGTAAAAT GTTAACAAAA GCTTAGTTAT	540
GTGACTGCTT GCGTATKTGT GATTCATTGA CTAGTTGKGT GTTACTACG GATGTTTAAC	600
AGGTCAAGGA GTAATGCTTG AGAAGCATAT TTAAGTTTTT ATTGTATGCA TGAATATCCA	660
GTAAGCATCA TAGAAAATGT AAAATTAANT TGTAAATAA TTAGAATACA TAGAAGAAAT	720
TGTTTAGATA AATATNATCT ATCTGAACAA TAAGGATGTC AGGATAGGAA AAGCTCTGTT	780
TCTGCAGCTT CCAGTGGAGA TCAGCACAGG AGGGAACCTA TTTTTT	826

WHAT IS CLAIMED IS:

- 1 1. A purified cytochrome P450 2C19 polypeptide
2 comprising an amino acid sequence having at least 97% sequence
3 identity with the amino acid sequence designated SEQ. ID.
4 No. 1.
- 1 2. A purified DNA segment encoding the purified
2 polypeptide of claim 1.
- 1 3. A stable cell line comprising an exogenous DNA
2 segment encoding a cytochrome P450 2C19 polypeptide of
3 claim 1, the DNA segment capable of being expressed in the
4 cell line.
- 1 4. A method of screening for a drug that is
2 metabolized by S-mephenytoin 4' hydroxylase activity, the
3 method comprising the steps of:
4 contacting the drug with a cytochrome P450 2C19
5 polypeptide of claim 1; and
6 detecting a metabolic product resulting from an
7 interaction between the drug and the polypeptide, the presence
8 of the product indicating the drug is metabolized by the S-
9 mephenytoin 4'-hydroxylase activity.
- 1 5. A method of diagnosing a patient having a
2 deficiency in S-mephenytoin 4'-hydroxylase activity, the
3 method comprising:
4 obtaining a sample of nucleic acids from the
5 patient; and
6 analyzing a cytochrome P450 2C19 DNA sequence
7 from the nucleic acids in the sample for the presence of a
8 polymorphism indicative of the deficiency.
- 1 6. The method of claim 5, further comprising the
2 step of amplifying the cytochrome P450 2C19 DNA sequence.
- 1 7. The method of claim 6, wherein the P450 2C19
2 DNA sequence is genomic.

1 8. The method of claim 7, wherein the amplifying
2 step is primed from a forward primer sufficiently
3 complementary with a first subsequence of the antisense strand
4 of the 2C19 sequence to hybridize therewith, and a reverse
5 primer sufficiently complementary to a second subsequence of
6 the sense strand of the 2C19 sequence to hybridize therewith.

1 9. The method of claim 8, wherein the polymorphism
2 occurs at nucleotide 681 of the coding region of the P450 2C19
3 DNA genomic sequence.

1 10. The method of claim 9, wherein the first
2 subsequence of the sense strand is upstream from nucleotide
3 681 of the coding region, and the second subsequence of the
4 antisense strand is downstream of nucleotide 681 of the coding
5 region.

1 11. The method of claim 10, wherein the analyzing
2 step comprises digesting the amplified DNA segment with a
3 restriction enzyme that recognizes a site including nucleotide
4 681 of the coding region.

1 12. The method of claim 8, wherein the polymorphism
2 occurs at nucleotide 636 of the coding region of the P450 2C19
3 DNA genomic sequence.

1 13. The method of claim 12, wherein the first
2 subsequence of the sense strand is upstream from nucleotide
3 636 of the coding region, and the second subsequence of the
4 antisense strand is downstream of nucleotide 636 of the coding
5 region.

1 14. The method of claim 13, wherein the analyzing
2 step comprises digesting the amplified DNA segment with a
3 restriction enzyme that recognizes a site including nucleotide
4 636 of the coding region.

1 15. The method of claim 8, wherein the polymorphism
2 occurs at nucleotide 636 or 681 of the coding region of the
3 P450 2C19 DNA genomic sequence, wherein the first subsequence
4 of the sense strand is upstream from nucleotide 636 of the
5 coding region, and the second subsequence of the antisense
6 strand is downstream of nucleotide 681 of the coding region.

1 16. The method of claim 9, wherein the forward
2 primer has
3 about 10-50 contiguous nucleotides from the
4 wildtype 2C19 sequence (SEQ. ID. No. 51) shown in Fig. 16
5 including the nucleotide at position 681 of the coding region;

6 wherein the forward primer primes amplification
7 from the complement of the wildtype 2C19 sequence without
8 priming amplification from the complement of the mutant 2C19
9 sequence shown in Fig. 16 (SEQ. ID. No. 61).

1 17. The method of claim 16, wherein the 3'
2 nucleotide of the forward primer is the nucleotide at position
3 681.

1 18. The method of claim 9, wherein the reverse
2 primer has
3 about 10-50 contiguous nucleotides from the
4 complement of the wildtype 2C19 sequence (SEQ. ID. No. 51)
5 shown in Fig. 16 including the complement to nucleotide 681 of
6 the coding region;
7 wherein the reverse primer primes amplification
8 from the wildtype 2C19 sequence without priming amplification
9 from the mutant 2C19 sequence (SEQ. ID. No. 61) shown in
10 Fig. 16.

1 19. The method of claim 18, wherein the 3'
2 nucleotide of the reverse primer is the complement of the
3 nucleotide at position 681.

1 20. The method of claim 9, wherein the forward
2 primer has
3 about 10-50 contiguous nucleotides from the
4 mutant 2C19 sequence shown in Fig. 16 including the nucleotide
5 at position 681 of the coding sequence,
6 wherein the forward primer primes amplification
7 from the complement of the mutant 2C19 sequence (SEQ. ID.
8 No. 61) without priming amplification from the complement of
9 the wildtype 2C19 (SEQ. ID. No. 51) sequence shown in Fig. 16.

1 21. The method of claim 20, wherein the 3'
2 nucleotide of the forward primer is the nucleotide at
3 position 681.

1 22. The method of claim 9, wherein the reverse
2 primer has
3 about 10-50 contiguous nucleotides from the
4 complement of the mutant 2C19 sequence (SEQ. ID. No. 61) shown
5 in Fig. 16 including the complement to nucleotide 681 of the
6 coding region;
7 wherein the reverse primer primes amplification
8 from the mutant 2C19 sequence without priming amplification
9 from the wildtype 2C19 (SEQ. ID. No. 51) sequence shown in
10 Fig. 16.

1 23. The method of claim 22, wherein the 3'
2 nucleotide of the reverse primer is the complement of the
3 nucleotide at position 681.

1 24. The method of claim 12, wherein the forward
2 primer has
3 about 10-50 contiguous nucleotides from the
4 wildtype 2C19 sequence (SEQ. ID. No. 52) shown in Fig. 17
5 including the nucleotide at position 636 of the coding region;
6 wherein the forward primer primes amplification
7 from the complement of the wildtype 2C19 sequence (SEQ. ID.
8 No. 54) without priming amplification from the complement of
9 the mutant 2C19 sequence shown in Fig. 17.

1 25. The method of claim 12, wherein the reverse
2 primer has
3 about 10-50 contiguous nucleotides from the
4 complement of the wildtype 2C19 sequence (SEQ. ID. No. 52)
5 shown in Fig. 17 including the complement to nucleotide 636 of
6 the coding region;
7 wherein the reverse primer primes amplification
8 from the wildtype 2C19 sequence without priming amplification
9 from the mutant 2C19 sequence (SEQ. ID. No. 54) shown in
10 Fig. 17.

1 26. The method of claim 12, wherein the forward
2 primer has
3 about 10-50 contiguous nucleotides from the
4 mutant 2C19 sequence (SEQ. ID. No. 54) shown in Fig. 17
5 including the nucleotide at position 636 of the coding
6 sequence,
7 wherein the forward primer primes amplification
8 from the complement of the mutant 2C19 sequence without
9 priming amplification from the complement of the wildtype 2C19
10 sequence (SEQ. ID. No. 52) shown in Fig 17.

1 27. The method of claim 12, wherein the reverse
2 primer has
3 about 10-50 contiguous nucleotides from the
4 complement of the mutant 2C19 sequence (SEQ. ID. No. 54) shown
5 in Fig. 17 including the complement to nucleotide 636 of the
6 coding region;
7 wherein the reverse primer primes amplification
8 from the mutant 2C19 sequence without priming amplification
9 from the wildtype 2C19 sequence (SEQ. ID. No. 52) shown in
10 Fig. 17.

1 28. The method of claim 6, wherein the segment of
2 the 2C19 sequence to be amplified is a cDNA sequence, and the
3 method further comprises the step of reverse transcribing mRNA
4 in the sample to produce the cDNA sequence.

1 29. The method of claim 28, wherein the forward
2 primer comprises about 10-50 contiguous nucleotides upstream
3 of nucleotide 643 of the coding region of the wildtype 2C19
4 cDNA sequence (SEQ. ID. No. 49) shown in Fig. 12 and
5 hybridizes to the complement of the 2C19 sequence upstream
6 from nucleotide 643 of the coding region, and the reverse
7 primer comprises about 10-50 contiguous nucleotides from the
8 complement of the wildtype 2C19 cDNA sequence (SEQ. ID No. 49)
9 shown in Fig. 12 and hybridizes to the 2C19 sequence
10 downstream from nucleotide 682 of the coding region.

1 30. The method of claim 28, wherein the forward
2 primer hybridizes to the complement of the wildtype 2C19 cDNA
3 sequence (SEQ. ID. No. 49) shown in Fig. 12 between
4 nucleotides 643 and 682 without hybridizing to the complement
5 of the mutant 2C19 cDNA sequence (SEQ. ID. No. 50) shown in
6 Fig. 12.

1 31. The method of claim 30, wherein the reverse
2 primer hybridizes to the wildtype 2C19 cDNA sequence (SEQ. ID.
3 No. 49) shown in Fig. 12 between nucleotides 643 and 682
4 without hybridizing to the mutant 2C19 cDNA sequence (SEQ. ID.
5 No. 50) shown in Fig. 12.

1 32. The method of claim 28, wherein the forward
2 primer comprises about 10-50 contiguous nucleotides upstream
3 of nucleotide 636 of the coding region of the wildtype 2C19
4 cDNA sequence (SEQ. ID. No. 49) shown in Fig. 12, and the
5 reverse primer comprises about 10-50 contiguous nucleotides
6 from the complement of the wildtype 2C19 cDNA sequence (SEQ.
7 ID. No. 49) shown in Fig. 12 downstream from nucleotide 636 of
8 the coding region.

1 33. The method of claim 28, wherein the full-length
2 2C19 cDNA sequence is amplified.

1 34. The method of claim 33, further comprising the
2 step of sequencing a segment of the 2C19 cDNA sequence.

1 35. The method of claim 5 further comprising the
2 step of:
3 digesting the DNA with a restriction enzyme
4 that recognizes a site including nucleotide 636 or 681 of the
5 2C19 DNA sequence;
6 wherein:
7 the 2C19 DNA sequence is genomic; and
8 the analyzing step comprises detecting the
9 products resulting from the digestion by Southern blotting
1 with a labelled segment of the 2C19 DNA sequence as a probe.

1 36. A diagnostic kit comprising:
2 a forward primer sufficiently complementary
3 with a first subsequence of the antisense strand of a double-
4 stranded 2C19 genomic DNA sequence to hybridize therewith, and
5 a reverse primer sufficiently complementary with a second
6 subsequence of the sense strand of the 2C19 genomic sequence
7 to hybridize therewith;
8 wherein the first subsequence is upstream of
9 nucleotide 681 of the coding region, and second subsequence is
10 downstream of nucleotide 681 of the coding region.

1 37. The diagnostic kit of claim 36, wherein the
2 first subsequence is upstream from nucleotide 636 of the
3 coding region.

1 38. The diagnostic kit of claim 36, wherein the
2 forward primer has about 10-50 contiguous nucleotides from the
3 wildtype 2C19 sequence (SEQ. ID. No. 51) shown in Fig. 16, and
4 the reverse primer has about 10-50 contiguous nucleotides from
5 the complement of the wildtype 2C19 sequence (SEQ. ID. No. 51)
6 shown in Fig. 16.

1 39. The diagnostic kit of claim 38, further
2 comprising

3 a second forward primer sufficiently
4 complementary with a first subsequence of the antisense strand
5 of a double-stranded 2C19 genomic DNA sequence to hybridize
6 therewith, and a a second reverse primer sufficiently
7 complementary with a second subsequence of the sense strand of
8 the 2C19 genomic sequence to hybridize therewith;

9 wherein the first subsequence is upstream of
10 nucleotide 636 of the coding region, and second subsequence is
11 downstream of nucleotide 636 of the coding region.

1 40. The diagnostic kit of claim 39, further
2 comprising a restriction enzyme that recognizes a site that
3 includes nucleotide 681 or nucleotide 636 of the coding
4 region.

1 41. A primer selected from the group consisting of:

2 (a) a first forward primer having:

3 about 10-50 contiguous nucleotides from
4 the wildtype 2C19 sequence (SEQ. ID. No. 51) shown in Fig. 16
5 including the nucleotide at position 681 of the coding region;

6 wherein the first forward primer primes
7 amplification from the complement of the wildtype 2C19
8 sequence without priming amplification from the complement of
9 the mutant 2C19 sequence (SEQ. ID. No. 61) shown in Fig. 16;

10 (b) a first reverse primer having:

11 about 10-50 contiguous nucleotides from
12 the complement of the wildtype 2C19 sequence (SEQ. ID. No. 51)
13 shown in Fig. 16 including the complement to nucleotide 681 of
14 the coding region;

15 wherein the first reverse primer primes
16 amplification from the wildtype 2C19 sequence without priming
17 amplification from the mutant 2C19 sequence shown in Fig. 16;

18 (c) a second forward primer having:

19 about 10-50 contiguous nucleotides from
20 the mutant 2C19 sequence (SEQ. ID. No. 61) shown in Fig. 16
21 including the nucleotide at position 681 of the coding sequence,

22 wherein the second forward primer primes
23 amplification from the complement of the mutant 2C19 sequence
24 without priming amplification from the complement of the
25 wildtype 2C19 sequence (SEQ. ID. No. 51) shown in Fig 16; and

26 (d) a second reverse primer having:
27 about 10-50 contiguous nucleotides from
28 the complement of the mutant 2C19 sequence (SEQ. ID. No. 61)
29 shown in Fig. 16 including the complement to nucleotide 681 of
30 the coding region;

31 wherein the second reverse primer primes
32 amplification from the mutant 2C19 sequence without priming
33 amplification from the wildtype 2C19 sequence (SEQ. ID.
34 No. 51) shown in Fig. 16

35 (e) a third forward primer having:
36 about 10-50 contiguous nucleotides from
37 the wildtype 2C19 sequence (SEQ. ID. No. 52) shown in Fig. 17
38 including the nucleotide at position 636 of the coding region;

39 wherein the first forward primer primes
40 amplification from the complement of the wildtype 2C19
41 sequence without priming amplification from the complement of
42 the mutant 2C19 sequence (SEQ. ID. No. 54) shown in Fig. 17;

43 (f) a third reverse primer having:
44 about 10-50 contiguous nucleotides from
45 the complement of the wildtype 2C19 sequence (SEQ. ID. No. 52)
46 shown in Fig. 17 including the complement to nucleotide 636 of
47 the coding region;

48 wherein the first reverse primer primes
49 amplification from the wildtype 2C19 sequence without priming
50 amplification from the mutant 2C19 sequence (SEQ. ID. No. 54)
51 shown in Fig. 17;

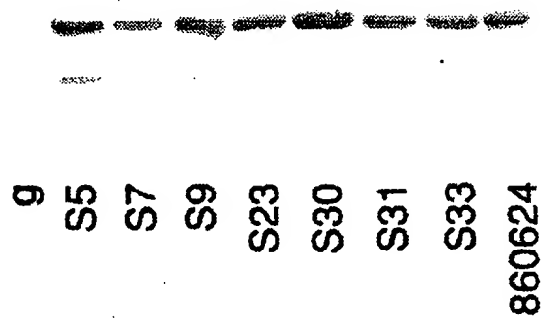
52 (g) a fourth forward primer having:
53 about 10-50 contiguous nucleotides from
54 the mutant 2C19 sequence (SEQ. ID. No. 54) shown in Fig. 17
55 including the nucleotide at position 636 of the coding
56 sequence,

57 wherein the second forward primer primes
58 amplification from the complement of the mutant 2C19 sequence

59 without priming amplification from the complement of the
60 wildtype 2C19 sequence (SEQ. ID. No. 52) shown in Fig 17; and
61 (h) a fourth reverse primer having:
62 about 10-50 contiguous nucleotides from
63 the complement of the mutant 2C19 sequence (SEQ. ID. No. 54)
64 shown in Fig. 17 including the complement to nucleotide 681 of
65 the coding region;
66 wherein the fourth reverse primer primes
67 amplification from the mutant 2C19 sequence without priming
68 amplification from the wildtype 2C19 sequence (SEQ. ID.
69 No. 52) shown in Fig. 17.

1/30

anti-ILC9



anti-ILC8

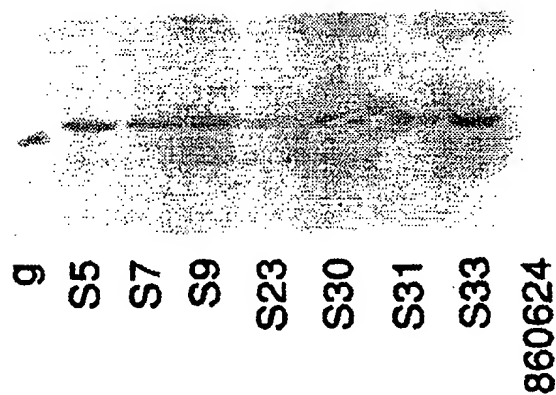


FIG. 1.

	-200	2/30				-150
2c	
2c8	
25	
65	
29c	.GGCACC	GGA AAGAACA	GGA AAAAAGA	ACA CCTTAT	TTTTT ATCTT	CCTTCA
6b	
11a	
	-151					-100
2c	
2c8	
25	
65	
29c	GTGAGC	CAAT GTTCAT	TCAA AAGAG	AGATT AAAGT	GCTTT TTGCT	GACTA
6b	
11a	
	-101					-50
2c	
2c8	
25	
65	
29c	GTCAC	AGTCA GAGTC	AGAAT CACAG	GTGGA TTAGT	AGGGA GTGTT	TATAAA
6b	
11a	
	-51					-1
2cAG	TGAAAG	CCCCG CAGTT	GTCTT ACTA	AGAAGA GAAG	.CTTCA
2c8A	
25GA	GAAGG	CTTCA
65	GAAGG	CTTCA
29c	AGCCTT	TGAAG	CCCCG CAGTT	GTCTT ACTA	AGAAGA GAAGC	CTTCA
6bAG	TGAAAG	CCCCG CAGTT	GTCTT ACTA	AGAAGA GAAGC	CTTCA
11aCTTCA	
	1					50
2c	ATGGAt	cCt. tTGTG	GtCCT .GTG	CtTGT CTCTC	aTgTt TGcTT	CtCtT
2c8	ATGGA	ACCTT TTGTG	GGTCCT GGTG	CtTGT CTCTC	TTTTA TGCTT	CtCtT
25	ATGGAT	TCTC TTGTG	GGTCCT TGTG	CtTGT CTCTC	ATGTT TGCTT	CtCtT
65	ATGGAT	TCTC TTGTG	GGTCCT TGTG	CtTGT CTCTC	ATGTT TGCTT	CtCtT
29c	ATGGAT	CCAG CTGTG	GGCTCT GGTG	CtTGT CTCTC	CTGTT TGCTT	CtCtT
6b	ATGGAT	CCAG CTGTG	GGCTCT GGTG	CtTGT CTCTC	CTGTT TGCTT	CtCtT
11a	ATGGAT	CCCTT TTGTG	GGTCCT TGTG	CtTGT CTCTC	ATGTT TGCTT	CtCtT

FIG. 2-1.

51		3/30		100	
2c	TTCACtCTGG	AGaCAGAGCT	cTgGgAGAgG	.Aa.CTCCct	cCTGGCCCCA
2c8	TTCACtCTGG	AGACAGAGCT	GTAGGAGAAG	GAAGCTCCCT	CCTGGCCCCA
25	TTCACtCTGG	AGACAGAGCT	CTGGGAGAGG	AAAACtCCCT	CCTGGCCCCA
65	TTCACtCTGG	AGACAGAGCT	CTGGGAGAGG	AAAACtCCCT	CCTGGCCCCA
29c	TTCACtCTGG	AGGCAGAGCT	CTGGAAGAGG	GAGGCTCCCG	TCTGGCCCCA
6b	TTCACtCTGG	AGGCAGAGCT	CTGGAAGAGG	GAGGCTCCCG	TCTGGCCCCA
11a	TTCAATCTGG	AGACAGAGCT	CTGGGAGAGG	AAAACtCCCT	CCTGGCCCCA
151				200	
2c	aGCAAATCct	TaACCAAT.T	CTCAAAagTC	TATGGcCCTG	TGTTCAct.T
2c8	TGCAAATCTT	TCACCAATTT	CTCAAAAGTC	TATGGTCCTG	TGTTCAccGT
25	AGCAAATCCT	TAACCAATCT	CTCAAAGGTC	TATGGCCCTG	TGTTCACTCT
65	AGCAAATCCT	TAACCAATCT	CTCAAAGGTC	TATGGCCCTG	TGTTCACTCT
29c	AGCAAATCCT	TAACCAATTT	CTCAAAAGTC	TATGGCCCTG	TGTTCACTGT
6b	AGCAAATCCT	TAACCAATTT	CTCAAAAGTC	TATGGCCCTG	TGTTCACTGT
11a	AGCAAATCCT	TAACCAATCT	CTCAAAAATC	TATGGCCCTG	TGTTCACTCT
201				250	
2c	GTATTTTGGC	cTGaAaCcCA	TaGTGGTG.T	gCATGGATAT	GAAGcaGTGA
2c8	GTATTTTGGC	ATGAATCCCA	TAGTGGTGTT	TCATGGATAT	GAGGCAGTGA
25	GTATTTTGGC	CTGAAACCCA	TAGTGGTGCT	GCATGGATAT	GAAGCAGTGA
65	GTATTTTGGC	CTGAAACCCA	TAGTGGTGCT	GCATGGATAT	GAAGCAGTGA
29c	GTATTTTGGC	CTGAAGCCCA	TTGTGGTGTT	GCATGGATAT	GAAGCAGTGA
11a	GTATTTTGGC	CTGGAACGCA	TGGTGGTGCT	GCATGGATAT	GAAGTGGTGA
251				300	
2c	AGGAAGCCCT	GATTGATc.T	GGAGAGGAGT	TTTCTGGAAG	AGGca.TTtc
2c8	AGGAAGCCCT	GATTGATAAT	GGAGAGGAGT	TTTCTGGAAG	AGGCAATTCC
25	AGGAAGCCCT	GATTGATCTT	GGAGAGGAGT	TTTCTGGAAG	AGGCATTTTC
65	AGGAAGCCCT	GATTGATCTT	GGAGAGGAGT	TTTCTGGAAG	AGGCATTTTC
29c	AGGAAGCCCT	GATTGATCAT	GGAGAGGAGT	TTTCTGGAAG	AGGAAGTTTT
6b	AGGAAGCCCT	GATTGATCAT	GGAGAGGAGT	TTTCTGGAAG	AGGAAGTTTT
11a	AGGAAGCCCT	GATTGATCTT	GGAGAGGAGT	TTTCTGGAAG	AGGCCATTTTC
301				350	
2c	CCACtGgCTg	AAAgAg.TAa	ca.AGGA.TT	GGAATcgTTT	tCAGCAATGG
2c8	CCAATATCTC	AAAGAATTAC	TAAAGGACTT	GGAATCATT	CCAGCAATGG
25	CCACTGGCTG	AAAGAGCTAA	CAGAGGATTT	GGAATTGTTT	TCAGCAATGG
65	CCACTGGCTG	AAAGAGCTAA	CAGAGGATTT	GGAATTGTTT	TCAGCAATGG
29c	CCAGTGGCTG	AAAAAGTTAA	CAAAGGACTT	GGAATCCTTT	TCAGCAATGG
6b	CCAGTGGCTG	AAAAAGTTAA	CAAAGGACTT	GGAATCCTTT	TCAGCAATGG
11a	CCACTGGCTG	AAAGAGCTAA	CAGAGGATTT	GGAATCGTTT	TCAGCAATGG

FIG. 2-2.

351		4/30		400	
2c	AAAGAGATGG	AAGGAGATCC	GGCGTTTCTc	CCTCAtgAcg	cTGCGGAATT
2c8	AAAGAGATGG	AAGGAGATCC	GGCGTTTCTC	CCTCACAAAC	TTGCGGAATT
25	AAAGAAATGG	AAGGAGATCC	GGCGTTTCTC	CCTCATGACG	CTGCGGAATT
65	AAAGAAATGG	AAGGAGATCC	GGCGTTTCTC	CCTCATGACG	CTGCGGAATT
29c	AAAGAGATGG	AAGGAGATCC	GGCGTTTCTG	CCTCATGACT	CTGCGGAATT
6b	AAAGAGATGG	AAGGAGATCC	GGCGTTTCTG	CCTCATGACT	CTGCGGAATT
11a	AAAGAGATGG	AAGGAGATCC	GGCGTTTCTC	CCTCATGACG	CTGCGGAATT
401				450	
2c	TTGGGATGGG	GAAGAGGAGC	ATtGAGGACC	GTGTTCAAGA	GGAAGCcCgC
2c8	TTGGGATGGG	GAAGAGGAGC	ATTGAGGACC	GTGTTCAAGA	GGAAGCTCAC
25	TTGGGATGGG	GAAGAGGAGC	ATTGAGGACC	GRGRRCAAGA	GGAAGCCCGC
65	TTGGGATGGG	GAAGAGGAGC	ATTGAGGACC	GTGTTCAAGA	GGAAGCCCGC
29c	TTGGGATGGG	GAAGAGGAGC	ATCGAGGACC	GTGTTCAAGA	GGAAGCCCGC
6b	TTGGGATGGG	GAAGAGGAGC	ATCGAGGACC	GTGTTCAAGA	GGAAGCCCGC
11a	TTGGGATGGG	GAAGAGGAGC	ATTGAGGACC	GTGTTCAAGA	GGAAGCCCGC
451				500	
2c	TGCCTTGTGG	AGGAGTTGAG	AAAAACCAAg	GCcTCACCCT	GTGATCCCAC
2c8	TGCCTTGTGG	AGGAGTTGAG	AAAAACCAAG	GCTTCACCCT	GTGATCCCAC
25	TGCCTTGTGG	AGGAGTTGAG	AAAAACCAAG	GCCTCACCCT	GTGATCCCAC
65	TGCCTTGTGG	AGGAGTTGAG	AAAAACCAAG	GCCTCACCCT	GTGATCCCAC
29c	TGCCTTGTGG	AGGAGTTGAG	AAAAACCAAT	GCCTCACCCT	GTGATCCCAC
6b	TGCCTTGTGG	AGGAGTTGAG	AAAAACCAAT	GCCTCACCCT	GTGATCCCAC
11a	TGCCTTGTGG	AGGAGTTGAG	AAAAACCAAG	GCTTCACCCT	GTGATCCCAC
501				550	
2c	TTTCATCCTG	GGCTGTGCTC	CCTGCAATGT	GATCTGCTCc	.TTaTTTTTCC
2c8	TTTCATCCTG	GGCTGTGCTC	CCTGCAATGT	GATCTGCTCC	GTTGTTTTTCC
25	TTTCATCCTG	GGCTGTGCTC	CCTGCAATGT	GATCTGCTCC	ATTATTTTTCC
65	TTTCATCCTG	GGCTGTGCTC	CCTGCAATGT	GATCTGCTCC	ATTATTTTTCC
29c	TTTCATCCTG	GGCTGTGCTC	CCTGCAATGT	GATCTGCTCT	GTTATTTTTCC
6b	TTTCATCCTG	GGCTGTGCTC	CCTGCAATGT	GATCTGCTCT	GTTATTTTTCC
11a	TTTCATCCTG	GGCTGTGCTC	CCTGCAATGT	GATCTGCTCC	ATTATTTTTCC
551				600	
2c	AtaAaCG.TT	tGATTATAAA	GATCAG.aaT	TTCTtAaCtT	gATGgAAAaa
2c8	AGAAACGATT	TGATTATAAA	GATCAGAATT	TTCTCACCCT	GATGAAAAGA
25	ATAAACGTTT	TGATTATAAA	GATCAGCAAT	TTCTTAACTT	AATGGAAAAG
65	ATAAACGTTT	TGATTATAAA	GATCAGCAAT	TTCTTAACTT	AATGGAAAAG
29c	ATGATCGATT	TGATTATAAA	GATCAGAGGT	TTCTTAACTT	GATGGAAAAA
6b	ATGATCGATT	TGATTATAAA	GATCAGAGGT	TTCTTAACTT	GATGGAAAAA
11a	AGAAACGTTT	CGATTATAAA	GATCAGCAAT	TTCTTAACTT	GATGGAAAAA

FIG. 2-3.

SUBSTITUTE SHEET (RULE 26)

601		5/30		650	
2c	TT.AATGAAA	ACaTCAGGAT	TcTgAgC.cc	CC.TGGATCC	AG.TcTGCAA
2c8	TTCAATGAAA	ACTTCAGGAT	TCTGAACTCC	CCATGGATCC	AGGTCTGCAA
25	TTGAATGAAA	ACATCAAGAT	TTTGAGCAGC	CCCTGGATCC	AGATCTGCAA
65	TTGAATGAAA	ACATCAAGAT	TTTGAGCAGC	CCCTGGATCC	AGATCTGCAA
29c	TTCAATGAAA	ACCTCAGGAT	TCTGAGCTCT	CCATGGATCC	AGGTCTGCAA
6b	TTCAATGAAA	ACCTCAGGAT	TCTGAGCTCT	CCATGGATCC	AGGTCTGCAA
11a	TTGAATGAAA	ACATCAGGAT	TGTAAGCACC	CCCTGGATCC	AGATATGCAA
651				700	
2c	TAATTT.cCt	cct.TCATTg	ATTattTCCC	.GGAActCA.	AACAAAtTac
2c8	TAATTTCCCT	CTACTCATTG	ATTGTTTCCC	AGGAACTCAC	AACAAAGTGC
25	TAATTTTCT	CCTATCATTG	ATTACTTCCC	GGGAACTCAC	AACAAATTAC
65	TAATTTTCT	CCTATCATTG	ATTACTTCCC	GGGAACTCAC	AACAAATTAC
29c	TAATTTCCCT	GCTCTCATCG	ATTATCTCCC	AGGAAGTCAT	AATAAAATAG
6b	TAATTTCCCT	GCTCTCATCG	ATTATCTCCC	AGGAAGTCAT	AATAAAATAG
11a	TAATTTTCCC	ACTATCATTG	ATTATTTCCC	GGGAACCCAT	AACAAATTAC
701				750	
2c	tTaAAAA.gT	TGCTtttAtg	aaAAGTtAta	TtttGGAgAa	AgTAAAAGAA
2c8	TTAAAAATGT	TGCTCTTACA	CGAAGTTACA	TTAGGGAGAA	AGTAAAAGAA
25	TTAAAAACGT	TGCTTTTATG	AAAAGTTATA	TTTTGGAAAA	AGTAAAAGAA
65	TTAAAAACGT	TGCTTTTATG	AAAAGTTATA	TTTTGGAAAA	AGTAAAAGAA
29c	CTGAAAATTT	TGCTTACATT	AAAAGTTATG	TATTGGAGAG	AATAAAAGAA
6b	CTGAAAATTT	TGCTTACATT	AAAAGTTATG	TATTGGAGAG	AATAAAAGAA
11a	TTAAAAACCT	TGCTTTTATG	GAAAGTGATA	TTTTGGAGAA	AGTAAAAGAA
751				800	
2c	CACCAAGaAT	Ca.TGGAcAt	gAACAA.cCT	CgGGACTTTA	TtGATTGcTT
2c8	CACCAAGCAT	CACTGGATGT	TAACAATCCT	CGGGACTTTA	TGGATTGCTT
25	CACCAAGAAT	CAATGGACAT	GAACAACCCT	CAGGACTTTA	TTGATTGCTT
65	CACCAAGAAT	CAATGGACAT	GAACAACCCT	CAGGACTTTA	TTGATTGCTT
29c	CATCAAGAAT	CCCTGGACAT	GAACAGTGCT	CGGGACTTTA	TTGATTGTTT
6b	CATCAAGAAT	CCCTGGACAT	GAACAGTGCT	CGGGACTTTA	TTGATTGTTT
11a	CACCAAGAAT	CGATGGACAT	CAACAACCCT	CGGGACTTTA	TTGATTGCTT
801				850	
2c	CCTGATcAAA	ATGGAg.AGG	AAAAGcAcAA	cCAAcagTcT	GAATTtAcTa
2c8	CCTGATCAAA	ATGGAGCAGG	AAAAGGACAA	CCAAAAGTCA	GAATTCAATA
25	CCTGATGAAA	ATGGAGAAGG	AAAAGCACAA	CCAACCATCT	GAATTTACTA
65	CCTGATGAAA	ATGGAGAAGG	AAAAGCACAA	CCAACCATCT	GAATTTACTA
29c	CCTGATCAAA	ATGGAACAGG	AAAAGCACAA	TCAACAGTCT	GAATTTACTG
6b	CCTGATCAAA	ATGGAACAGG	AAAAGCACAA	TCAACAGTCT	GAATTTACTG
11a	CCTGATCAAA	ATGGAGAAGG	AAAAGCAAAA	CCAACAGTCT	GAATTCACTA

FIG. 2-4.

851		6/30		900	
2c	TTGAAAgCTT	Ggta..CACT	G.AgcTGA.t	TgtTTGgaGC	TGG.ACAGAG
2c8	TTGAAAACCTT	GGTTGGCACT	GTAGCTGATC	TATTTGTTGC	TGGAACAGAG
25	TTGAAAGCTT	GGAAAACACT	GCAGTTGACT	TGTTTGGAGC	TGGGACAGAG
65	TTGAAAGCTT	GGAAAACACT	GCAGTTGACT	TGTTTGGAGC	TGGGACAGAG
29c	TTGAAAGCTT	GATAGCCACT	GTAAGTATA	TGTTTGGGGC	TGGAACAGAG
6b	TTGAAAGCTT	GATAGCCACT	GTAAGTATA	TGTTTGGGGC	TGGAACAGAG
11a	TTGAAAACCTT	GGTAATCACT	GCAGCTGACT	TACTTGGAGC	TGGGACAGAG
901				950	
2c	ACaACaAGCA	C.AC.CTGAG	ATATG..CTC	CT.CTCCTGC	TGAAGcACCC
2c8	ACAACAAGCA	CCACTCTGAG	ATATGGACTC	CTGCTCCTGC	TGAAGCACCC
25	ACGACAAGCA	CAACCCTGAG	ATATGCTCTC	CTTCTCCTGC	TGAAGCACCC
65	ACGACAAGCA	CAACCCTGAG	ATATGCTCTC	CTTCTCCTGC	TGAAGCACCC
29c	ACAACGAGCA	CCACTCTGAG	ATATGGACTC	CTGCTCCTGC	TGAAGTACCC
6b	ACAACGAGCA	CCACTCTGAG	ATATGGACTC	CTGCTCCTGC	TGAAGTACCC
11a	ACAACAAGCA	CAACCCTGAG	ATATGCTCTC	CTTCTCCTGC	TGAAGCACCC
951				1000	
2c	AGAGGTCACA	GCTAAAGTCC	AGGAAGAGAT	TGAacgTGta	aTTGGCAGAA
2c8	AGAGGTCACA	GCTAAAGTCC	AGGAAGAGAT	TGATCATGTA	ATTGGCAGAC
25	AGAGGTCACA	GCTAAAGTCC	AGGAAGAGAT	TGAACGTGTG	ATTGGCAGAA
65	AGAGGTCACA	GCTAAAGTCC	AGGAAGAGAT	TGAACGTGTG	ATTGGCAGAA
29c	AGAGGTCACA	GCTAAAGTCC	AGGAAGAGAT	TGAATGTGTA	GTTGGCAGAA
6b	AGAGGTCACA	GCTAAAGTCC	AGGAAGAGAT	TGAATGTGTA	GTTGGCAGAA
11a	AGAGGTCACA	GCTAAAGTCC	AGGAAGAGAT	TGAACGTGTC	ATTGGCAGAA
1001				1050	
2c	ACcGGAGCCC	CTGcATGCag	GAcAGGaGcC	ACATGCCcTA	CACaGATGCT
2c8	ACAGGAGCCC	CTGCATGCAG	GATAGGAGCC	ACATGCCTTA	CACTGATGCT
25	ACCGGAGCCC	CTGCATGCAA	GACAGGAGCC	ACATGCCCTA	CACAGATGCT
65	ACCGGAGCCC	CTGCATGCAA	GACAGGAGCC	ACATGCCCTA	CACAGATGCT
29c	ACCGGAGCCC	CTGTATGCAG	GACAGGAGTC	ACATGCCCTA	CACAGATGCT
6b	ACCGGAGCCC	CTGTATGCAG	GACAGGAGTC	ACATGCCCTA	CACAGATGCT
11a	ACCGGAGCCC	CTGCATGCAG	GACAGGGGCC	ACATGCCCTA	CACAGATGCT
1051				1100	
2c	GTgGTGCACG	AG.TCCAGAG	ATACattGAC	CT.cTCCCCA	CCagccTGCC
2c8	GTAGTGCACG	AGATCCAGAG	ATACAGTGAC	CTTGTCCCCA	CCGGTGTGCC
25	GTGGTGCACG	AGGTCCAGAG	ATACCTTGAC	CTTCTCCCCA	CCAGCCTGCC
65	GTGGTGCACG	AGGTCCAGAG	ATACATTGAC	CTTCTCCCCA	CCAGCCTGCC
29c	GTGGTGCACG	AGATCCAGAG	ATACATTGAC	CTCCTCCCCA	CCAACCTGCC
6b	GTGGTGCACG	AGATCCAGAG	ATACATTGAC	CTCCTCCCCA	CCAACCTGCC
11a	GTGGTGCACG	AGGTCCAGAG	ATACATCGAC	CTCATCCCCA	CCAGCCTGCC

FIG. 2-5.

SUBSTITUTE SHEET (RULE 26)

	1101		7/30		1150
2c	CCATGCAGTG	ACCTgTGA..	tTAAaTTCAG	AAACTAcCTC	AT.CCCCAAGG
2c8	CCATGCAGTG	ACCACTGATA	CTAAGTTCAG	AAACTACCTC	ATCCCCAAGG
25	CCATGCAGTG	ACCTGTGACA	TTAAATTTCAG	AAACTATCTC	ATTCCCCAAGG
65	CCATGCAGTG	ACCTGTGACA	TTAAATTTCAG	AAACTATCTC	ATTCCCCAAGG
29c	CCATGCAGTG	ACCTGTGATG	TTAAATTCAA	AAACTACCTC	ATCCCCAAGG
6b	CCATGCAGTG	ACCTGTGATG	TTAAATTCAA	AAACTACCTC	ATCCCCAAGG
11a	CCATGCAGTG	ACCTGTGACG	TTAAATTTCAG	AAACTACCTC	ATTCCCCAAGG
	1151				1200
2c	GCACaACCAT	A.Taac.Tcc	CTgACTTCTg	TGCTaCatgA	..ACAAAGAA
2c8	GCACAACCAT	AATGGCATTa	CTGACTTCCG	TGCTACATGA	TGACAAAGAA
25	GCACAACCAT	ATTAATTTCC	CTGACTTCTG	TGCTACATGA	CAACAAAGAA
65	GCACAACCAT	ATTAATTTCC	CTGACTTCTG	TGCTACATGA	CAACAAAGAA
29c	GCACgACCAT	AATAACATCC	CTGACTTCTG	TGCTGCACAA	TGACAAAGAA
6b	GCATGACCAT	AATAACATCC	CTGACTTCTG	TGCTGCACAA	TGACAAAGAA
11a	GCACAACCAT	ATTAACTTCC	CTCACTTCTG	TGCTACATGA	CAACAAAGAA
	1201				1250
2c	TTtCCcAAcC	CAGAgATgTT	TGACCCT.g.	CACTTTCTgG	AT.A..gTGG
2c8	TTTCCTAATC	CAAATATCTT	TGACCCTGGC	CACTTTCTAG	ATAAGAATGG
25	TTTCCCAACC	CAGAGATGTT	TGACCCTCAT	CACTTTCTGG	ATGAAGGTGG
65	TTTCCCAACC	CAGAGATGTT	TGACCCTCAT	CACTTTCTGG	ATGAAGGTGG
29c	TTCCCAACC	CAGAGATGTT	TGACCCTGGC	CACTTTCTGG	ATAAGAGTGG
6b	TTCCCAACC	CAGAGATGTT	TGACCCTGGC	CACTTTCTGG	ATAAGAGTGG
11a	TTTCCCAACC	CAGAGATGTT	TGACCCTCGT	CACTTTCTGG	ATGAAGGTGG
	1251				1300
2c	cAA.TTTAAG	AAAAGT.AcT	ACTTCATGCC	TTTCTCAGCA	GGAAAACGcA
2c8	CAACTTTAAG	AAAAGTGACT	ACTTCATGCC	TTTCTCAGCA	GGAAAACGAA
25	CAATTTTAAG	AAAAGTAAAT	ACTTCATGCC	TTTCTCAGCA	GGAAAACGGA
65	CAATTTTAAG	AAAAGTAAAT	ACTTCATGCC	TTTCTCAGCA	GGAAAACGGA
29c	CAACTTTAAG	AAAAGTGACT	ACTTCATGCC	TTTCTCAGCA	GGAAAACGGA
6b	CAACTTTAAG	AAAAGTGACT	ACTTCATGCC	TTTCTCAGCA	GGAAAACGGA
11a	AAATTTTAAG	AAAAGTAACT	ACTTCATGCC	TTTCTCAGCA	GGAAAACGGA
	1301				1350
2c	TtTGTgtgGG	AGA>GgcCTg	GCCcGCATGG	AGCTgTTTTT	ATTcCTgACC
2c8	TTTGTGCAGG	AGAAGGACTT	GCCCGCATGG	AGCTATTTTT	ATTCTAACC
25	TTTGTGTGGG	AGAAGCCCTG	GCCGGCATGG	AGCTGTTTTT	ATTCCTGACC
65	TTTGTGTGGG	AGAAGCCCTG	GCCGGCATGG	AGCTGTTTTT	ATTCCTGACC
29c	TGTGTATGGG	AGAGGGCCTG	GCCCGCATGG	AGCTGTTTTT	ATTCCTGACC
6b	TGTGTATGGG	AGAGGGCCTG	GCCCGCATGG	AGCTGTTTTT	ATTCCTGACC
11a	TTTGTGTGGG	AGAGGGCCTG	GCCCGCATGG	AGCTGTTTTT	ATTCCTGACC

FIG. 2-6.

	1351		8/30		1400
2c	.ccATTTTaC	AGAACTTTAA	CCTGAAATCT	ctggtTGAcc	cAAAG.AccT
2c8	ACAATTTTAC	AGAACTTTAA	CCTGAAATCT	GTTGATGATT	TAAAGAACCT
25	TCCATTTTAC	AGAACTTTAA	CCTGAAATCT	CTGGTTGACC	CAAAGAACCT
65	TCCATTTTAC	AGAACTTTAA	CCTGAAATCT	CTGGTTGACC	CAAAGAACCT
29c	ACCATTTTGC	AGAACTTTAA	CCTGAAATCT	CAGGTTGACC	CAAAGGATAT
6b	ACCATTTTGC	AGAACTTTAA	CCTGAAATCT	CAGGTTGACC	CAAAGGATAT
11a	TTCATTTTAC	AGAACTTTAA	CCTGAAATCT	CTGATTGACC	CAAAGGACCT
	1401				1450
2c	tgAcAccACT	cCagTTg.CA	AtGgatTTGc	ttcTgTgCC.	CCCTtcTAcC
2c8	CAATACTACT	GCAGTTACCA	AAGGGATTGT	TTCTCTGCCA	CCCTCATACC
25	TGACACCACT	CCAGTTGTCA	ATGGTTTTCG	CTCTGTGCCG	CCCTTCTACC
65	TGACACCACT	CCAGTTGTCA	ATGGATTTCG	CTCTGTGCCG	CCCTTCTACC
29c	TGACATCACC	CCCATTGCCA	ATGCATTTGG	TDGTGTGCCA	CCCTTGTACC
6b	TGACATCACC	CCCATTGCCA	ATGCATTTGG	TCGTGTGCCA	CCCTTGTACC
11a	TGACACAACCT	CCTGTTGTCA	ATGGATTTCG	TTCTGTCCCG	CCCTTCTATC
	1451		***		1500
2c	AGcT.TGCTT	CATtCCTGTC	TGAAGAAggg	cAGatggtcT	GGCTGCT.cT
2c8	AGATCTGCTT	CATCCCTGTC	TGAAGAATGC	TAGCCCATCT	GGCTGCTGAT
25	AGCTGTGCTT	CATTCCTGTC	TGAAGAAGAG	CAGATGGCCT	GGCTGCTGCT
65	AGCTGTGCTT	CATTCCTGTC	TGAAGAAGAG	CAGATGGCCT	GGCTGCTGCT
29c	AGCTCTGCTT	CATTCCTGTC	TGAAGAAGGG	CAGATAGTTT	GGCTGCTCCT
6b	AGCTCTGCTT	CATTCCTGTC	TGAAGAAGGG	CAGATAGTTT	GGCTGCTCCT
11a	AGCTGTGCTT	CATTCCTGTC	TGAAGAAGCA	CAGATGGTCT	GGCTGCTCCT
	1501				1550
2c	gTGctgTC.Ct...	ttt..tctgg	ggcaatttcC	.tctt.cat.
2c8	CTGCTATCAC	CTGCAACTCT	TTTTTTATCA	AGGACATTCC	CACtATTATG
25	GTGCAGTCCC	TGCAGCTCTC	TTTCCTCTGG	GGCATTATCC	ATCTTTCACT
65	GTGCAGTCCC	TGCAGCTCTC	TTTCCTCTGG	GGCATTATCC	ATCTTTCACT
29c	GTGCTGTCAC	CTGCAATTCT	CCCTTATCAG	GGCCATTAGC	CTCTCCCTTC
6b	GTGCTGTCAC	CTGCAATTCT	CCCTTATCAG	GGCCATTGGC	CTCTCCCTTC
11a	GTGCTGTCCC	TGCAGCTCTC	TTTCCTCTGG	TCCAAATTTC	ACTATCTGTG
	1551				1600
2c	..t.tt..tg	c..ttt.Tca	tcTg.catct	caca.t.c..	cttccctta.
2c8	TCTTCTCTGA	CCTCTCATCA	AATCTTCCCA	TTCACtCAAT	ATCCCATAAG
25	ATCTGTAATG	CCTTTTCTCA	CCTGTCATCT	CACATTTTCC	CTTCCCTGAA
65	ATCTGTAATG	CCTTTTCTCA	CCTGTCATCT	CACATTTTCC	CTTCCCTGAA
29a	TCTCTGTGAG	GGATATTTTC	TCTGACTTGT	CAATCCACAT	CTTCCCATTC
6b	TCTCTATGAG	GGATATTTTC	TCTGACTTGT	CAATCCACAT	CTTCCCATTC
11a	ATGCTTCTTC	TGACCCGTCA	TCTCACATTT	TCCCTTCCCC	CAAGATCTAG

FIG. 2-7.

9/30

1601 1650

2c catc.Ag..a ccaTt.a... .caat.tcca agag.gtg.. ttt.Tt..ct
 2c8 CATCCAAACT CCATTAAGGA GAGTTGTTCA GGTCAC TGCA CAAATATATC
 25 GATCTAGTGA ACATTCGACC TTCATTACGG AGAGTTTCCT ATGTTTCACT
 65 GATCTAGTGA ACATTCGACC TCCATTACTT AGAGTTTCCT ATGTTTCACT
 29c CCTCAAGATC CAATGAACAT CCAACCTCCA TTAAAGAGAG TTTCTTGGGT
 6b CCTCAAGATC CAATGAACAT CCAACCTCCA TTAAAGAGAG TTTCTTGGGT
 11a TGAACATTCA GCCTCCATTA AAAAAGTTTC ACTGTGCAAA TATATCTGCT

1651 1700

2c .tccaccta. atctatc..tct.ct. t.t.t..aT. actttgattg
 2c8 TGCAATTATT CATACTCTGT AACACTTGTA TTAATTGCTG CATATGCTAA
 25 GTGCAAATAT ATCTGCTATT CTCCATACTC TGTAACAGTT GCATTGACTG
 65 GTGCAAATAT ATCTGCTATT CTCCATACTC TGTAACAGTT GCATTGACTG
 29c CACTTCCTAA ATATATCTGC TATTCTCCAT ACTCTGTATC ACTTGTATTG
 6b CACTTCCTAA ATATATCTGC TATTCTCCAT ACTCTGTATC ACTTGTATTG
 11a ATTCCCCATA CTCTATAATA GTTACATTGA GTGCCACATA ATGCTGATAC

1701 1750

2c tcc.cta.tg aTg.taatt. tttaatattg ..ttattg.. A...t.ttAt
 2c8 TACTTTTCTA ATGCTGACTT TTTAATATGT TATCACTGTA AAACACAGAA
 25 TCACATAATG CTCATACTTA TCTAATGTTG AGTTATTAAT ATGTTATTAT
 65 TCACATAATG CTCATACTTA TCTAATGTTG AGTTATTAAT ATGTTATTAT
 29c ACCACCACAT ATGCTAATAC CTATCTACTG CTGAGTTGTC AGTATGTTAT
 6b ACCACCACAT ATGCTAATAC CTATCTACTG CTGAGTTGTC AGTATGTTAT
 11a TTGTCTAATG TTGAGTTATT AACATATTAT TATTAAATAG A

1751 1800

2c .A.t.a.aaA .aaAtgAtaa tt.t.t..aa aT...aagtc A.tgc..tt.
 2c8 AAGTGATTAA TGAATGATAA TTTAGTCCAT TTCTTTTGTG AATGTGCTAA
 25 TAAATAGAGA AATATGATTT GTGTATTATA ATTCAAAGGC ATTTCTTTTC
 65 TAAATAGAGA AATATGATTT GTGTATTATA ATTCAAAGGC ATTTCTTTTC
 29c CACTAGAAAA CAAAGAAAAA TGATTAATAA ATGACAATTC AGAGCCAAAA
 6b CACTATAAAA CAAAGAAAAA TGATTAATAA ATGACAATTC AGAGCCATTT

1801 1850

2c a..at.t.c. .aaTaaAaag cattaTtATT tgctgaaAaa aaGTCAGTTC
 2c8 ATAAAAAGTG TTATTAATTG CTGGTTCA
 25 TGCATGTTCT AAATAAAAAG CATTATTATT TGCTGAAAAA AA
 65 TGCATGTTCT AAATAAAAAG CATTATTATT TGCTGAAAAA AA
 29c AAAAAAAAAA
 6b ATTCTCTGCA TGCTCTAGAT AAAAATGATT ATTATTTACT GGGTCAGTTC

FIG. 2-8.

10/30

1851 1900
6b TTAGATTTCT TTCTTTTGAG TAAATGAAA GTAAGAAATG AAAGAAAATA

1901 1950
6b GAATGTGAAG AGGCTGTGCT GGCCTCATA GTGTTAAGCA CAAAAGGGA

1951 2000
6b GAAAGGTAAG AGGGTAGGAA AGCTGTTTTA GCTAAATGCC ACCTAGAGTT

2001 2050
6b ATTGGAGGTC TGAATTGGA AAAAAAACT ATGTCCAGGA GAACATTAAG

2101 2150
6b TGTTTGAATT CATGCTCTGC TTTTGTGTTA CTGTAAACAC AAGATCAAGA

2151 2200
6b TTTGGATAAT CTTTTTCCTT TGTGTTTCCA ACTTAGATCA TGTCTAAATA

2201 2216
6b TATGCTTTCA TATGGC

FIG. 2-9.

II/30

1	MDp.VvLVLC	LScLlLlslw	RQSSgRgkLP	pGPTPLP.IG	NIQId.KDi	sKsLTN.SKv	YGPVFT.YFG	70
IIC8	MEPFVVLVLC	LSFMLlFSLW	RQSCRRRKLP	PGPTPLPIIG	NMLQIDVKDI	CKSFTNFskv	YGPVFTVYFG	
65	MDSl.VVLVLC	LScLlLlLslw	RQSSGRGKLP	PGPTPLPVIG	NIQIGIKDI	SKSl.TNLSKV	YGPVFTl.YFG	
25	MDSl.VVLVLC	LScLlLlLslw	RQSSGRGKLP	PGPTPLPVIG	NIQIGIKDI	SKSl.TNLSKV	YGPVFTl.YFG	
29c	MDPAVALVLC	LScLFLLSlw	RQSSGRGRIP	SGPTPLPIIG	NIQLDVKDM	SYSI.TNFskU	TGPVGTVYFG	
6b	MDPAVALVLC	LScLFLLSlQ	RQSSGRGRIP	SGPTPLPIIG	NIQLDVKDM	SKSl.TNFskv	YGPVFTVYFG	
11a	MDPFVVLVLC	LScLlLlSlQ	RQSSGRGKLP	PGPTPLPVIG	NIQIDIKDV	SYSLTNLSKI	YGPVFTl.YFG	
/1								140
	IkpiVVLHGy	EaVKEALIDl	GEeFSGRG.f	Plaeran.G.	GIVfSNGKrw	KEIRREsLmt	LRNFGMGKRS	
IIC8	MNPiVVLHGy	EaVKEALIDN	GEeFSGRGNS	PISQRITKGL	GISSNGKRW	KEIRRFSLTN	LTNFGMGKRS	
65	IKPiVVLHGy	EaVKEALIDl	GEeFSGRGIF	PLAERANRGF	GIVfSNGKKW	KEIRRFSLMT	LRNFGMGKRS	
25	IKPiVVLHGy	EaVKEALIDl	GEeFSGRGIF	PLAERANRGF	GIVfSNGKKW	KEIRRFSLMT	LRNFGMGKRS	
29c	IKPiVVLHGy	EaVKEALIDH	GEeFSGRGSF	PVAEKVNKGL	GILfSNGKRW	KEIRRFCLMT	LRNf'GMGKRS	
6b	IKPiVVLHGy	EaVKEALIDH	GEeFSGRGSF	PVAEKVNKGL	GILfSNGKRW	KEIRRFCLMT	LRNFGMGKRS	
11a	IERMVVLHGy	EvvKEALIDl	GEeFSGRGHF	PLAERANRGF	GIVfSNGKRW	KEIRRFSLMT	LRNFGMGKRS	
141								210
	IEDRVQEEAr	CLVEELRKTK	ASPCDPTFiL	GCAPCNVICS	iiFhkRFDYK	DQqFLnLMek	INENirIlss	
IIC8	IEDRVQEEAH	CLVEELRKTK	ASPCDPTFiL	GCAPCNVICS	VVFQKRFDYK	DQNFLTLMKR	FNENFRILNS	
65	IEDRVQEEAR	CLVEELRKTK	ASPCDPTFiL	GCAPCNVICS	IIFHKRFDYK	DQqFLNLMEK	LNENIKILSS	
25	IEDRVQEEAR	CLVEELRKTK	ASPCDPTFiL	GCAPCNVICS	IIFHKRFDYK	DQqFLNLMEK	LNENIKILSS	
29c	IEDRVQEEAR	CLVEELRKTN	ASPCDPTFiL	GCAPCNVICS	VIFHdRFDYK	DQRFNLMEK	FNENLRILSS	
6b	IEDRVQEEAR	CLVEELRKTN	ASPCDPTFiL	GCAPCNVICS	VIFHdRFDYK	DQRFNLMEK	FNENLRILSS	
11a	IEDRVQEEAR	CLVEELRKTK	ASPCDPTFiL	GCAPCNVICS	IIFQKRFDYK	DQqFLNLMEK	LNENIRIVST	

FIG. 3-1.

12/30

```

211
1 IC8 PWIQICNNFP .iIDYfPGtH NKllKnvaFm kSYilekvKE HQeSlDmNnp rDFiDCFLik MEqEKhnQqS 280
65 PWIQICNNFP .lIDCFPGtH NKVLKNVAI.T RSYIREKVKE HQASLDVNNP RDFMDCFLik MEDEKDNQKS
25 PWIQICNNFP .PIIDYfPGtH NKLLKNVAFM KSYILEKVKE HQESMDMNNP QDFIDCFLMK MEKEKHNPQS
29c PWIQICNNFP .PIIDYfPGtH NKLLKNVAFM KSYILEKVKE HQESMDMNNP QDFIDCFLMK MEKEKHNPQS
6b PWIQICNNFP .ALIDYLPgSH NKIAENFAYI KSYVLERIKE HQESLDMNSA RDFIDCFlik MEQEKHNQQS
11a PWIQICNNFP .TIIDYfPGtH NKLLKNLAFM ESDILEKVKE HQESMDINNp RDFIDCFlik MEQEKHNQQS
281
1 IC8 EftiEsLiAt vtdlfgAGTE tTSTTLRYaL LLLLKhPEVT AKVQEEIerv iGRnRSPCMQ DRsHMPYTDA 350
65 EFTIESLENT VADLFVAGTE TTSTTLRYGL LLLLKHPEVT AKVQEEIDHV IGRHRSPCMQ DRSHMPYTDA
25 EFTIESLENT AVDLFGAGTE TTSTTLRYAL LLLLKHPEVT AKVQEEIERV IGRNRSPCMQ DRSHMPYTDA
29c EFTVESLIAT VTDMFGAGTE TTSTTLRYGL LLLLYPEVT AKVQEEIECV VGRNRSPCMQ DRSHMPYTDA
6b EFTVESLIAT VTDMFGAGTE TTSTTLRYGL LLLLYPEVT AKVQEEIECV VGRNRSPCMQ DRSHMPYTDA
11a EFTIENLVIT AADLLGAGTE STSTTLRYAL LLLLKHPEVT AKVQEEIERV IGRNRSPCMQ DRGHMPYTDA
351
1 IC8 VVHEVQRYID LIPTslPHAV TcdVKEFrNYL IPKGtTilts LTSVLHdnKE FPNPemFDpG HFLDegGNEK 420
65 VVHEIQRYSD LVPTGVPHAV TTDTKFRNYL IPKGTTIMAl LTSVLHDDKE FPNPIFDpG HFLDKNGNEK
25 VVHEVQRYID LLPTSLPHAV TCDIKFRNYL IPKGTTILIS LTSVLHDNKE FPNPEMFDPH HFLDEFFNEK
29c VVHEVQRYID LLPTSLPHAV TCDIKFRNYL IPKGTTILIS LTSVLHDNKE FPNPEMFDPH HFLDEFFNEK
6b VVHEIQRYID LLPTNLPHAV TCDVKEKNYL IPKGTTIITS LTSVLHNDKE FPNPEMFDPG HFLDKSGNEK
11a VVHEVQRYID LIPTSLPHAV TCDVKEFRNYL IPKGTTILTS LTSVLHDNKE FPNPEMFDPG HELDKSGNEK

```

FIG. 3-2.

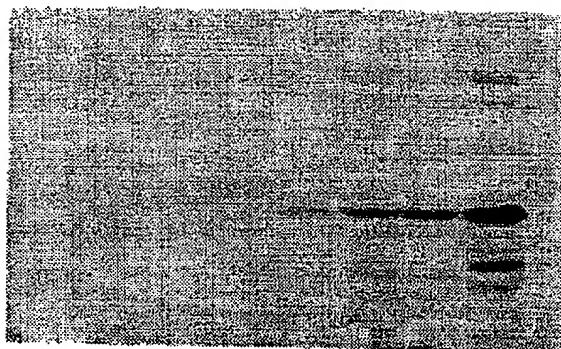
13/30

421 KSDYFMPFSA GKRICVGEGL ARMEFLFELT TILQNFNLKS LVDPKDLDTT PVVNGFASVP PFYCLCFIPV *
 IIC8 KSDYFMPFSA GKRICAGEGL ARMEFLFELT TILQNFNLKS VDDLKNLNTT AVTKGIVSLP PSYCICFIPV *
 65 KSKYFMPFSA GKRICVGEAL AGMEFLFELT SILQNFNLKS LVDPKNLD'TT PVVNGFASVP PFYCLCFIPV *
 25 KSKYFMPFSA GKRICVGEAL AGMEFLFELT SILQNFNLKS LVDPKNLD'TT PVVNGFASVP PFYCLCFIPV *
 29c KSDYFMPESA GKRMCMGEGL ARMEFLFELT TILQNFNLKS QVDPKDIDIT PIANAFGRVP PLYCLCFIPV *
 6b KSDYFMPESA GKRMCMGEGL ARMEFLFELT TILQNFNLKS QVDPKDIDIT PIANAFGRVP PLYCLCFIPV *
 11a KSNYFMPESA GKRICVGEGL ARMEFLFELT TILQNFNLKS LIDPKDLDTT PVVNGFASVP PFYCLCFIPV *

FIG. 3-3.

14/30

anti-ILC9



con 6b 29c 11a 25 65 65 S5 9

anti-ILC8



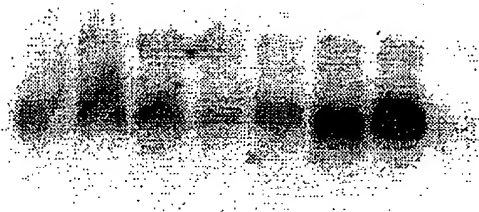
con 6b 29c 11a 25 65 65 S5 9

FIG. 4.

15/30

T300R

860624
S33
S31
S30
S23
S9
S7
S5



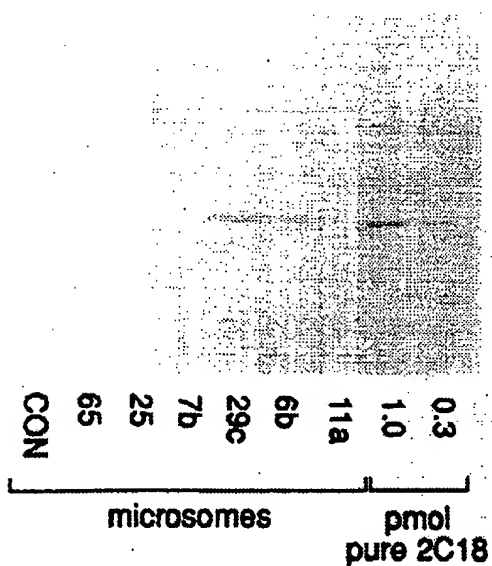
Actin



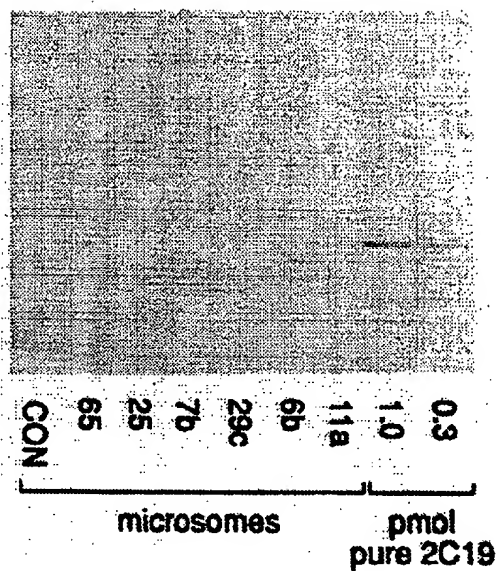
FIG. 5.

16/30

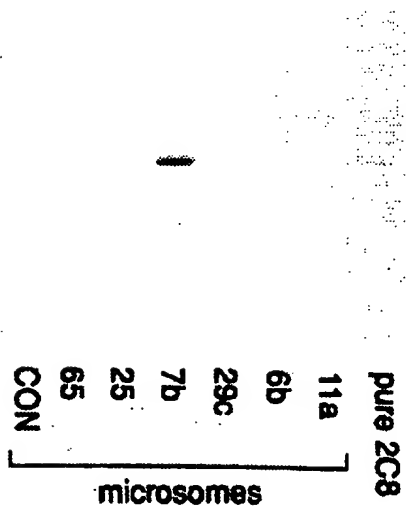
Anti - 2C18



Anti - 2C19



Anti - 2C8



Anti - 2C9

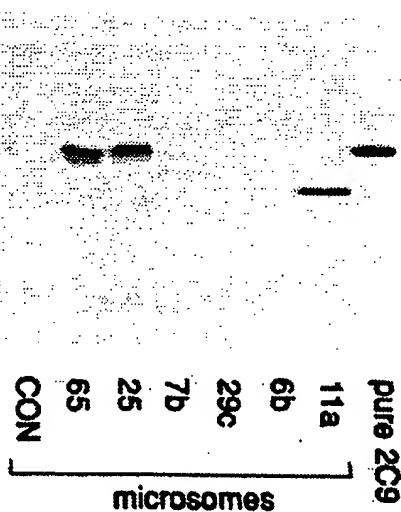
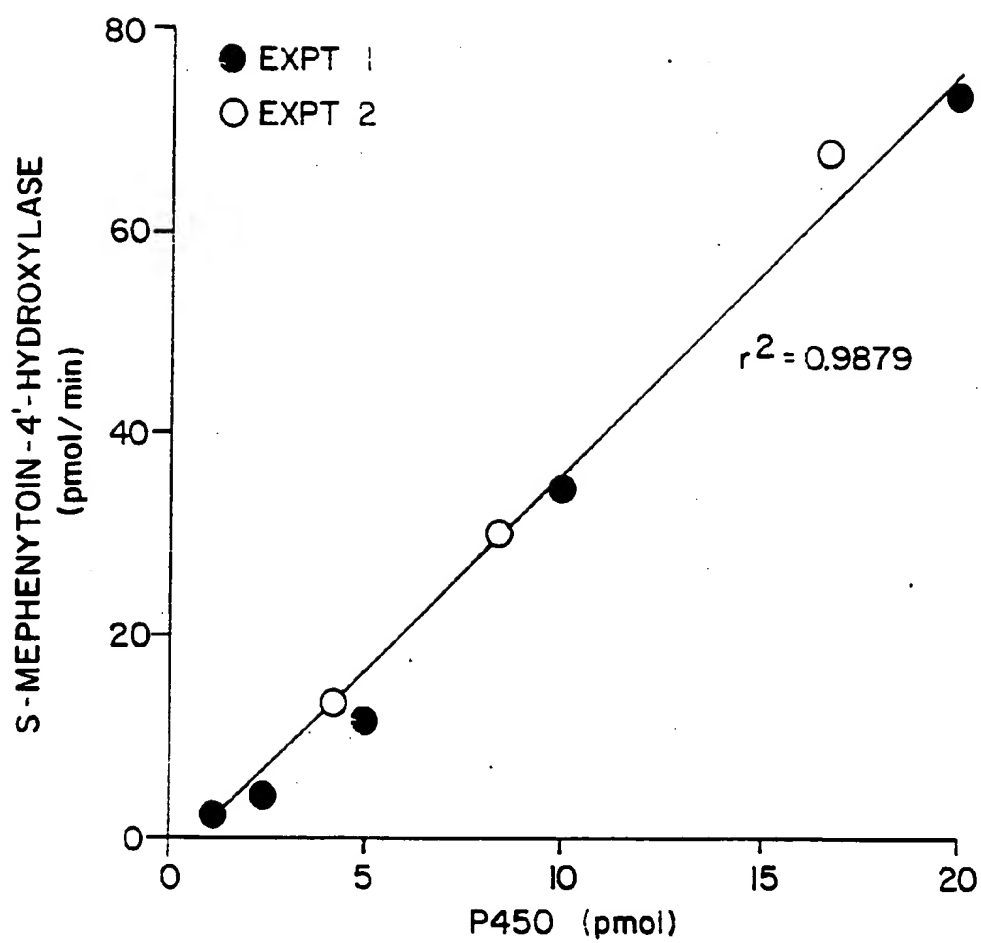


FIG. 6.

SUBSTITUTE SHEET (RULE 26)

17/30

**FIG. 7.**

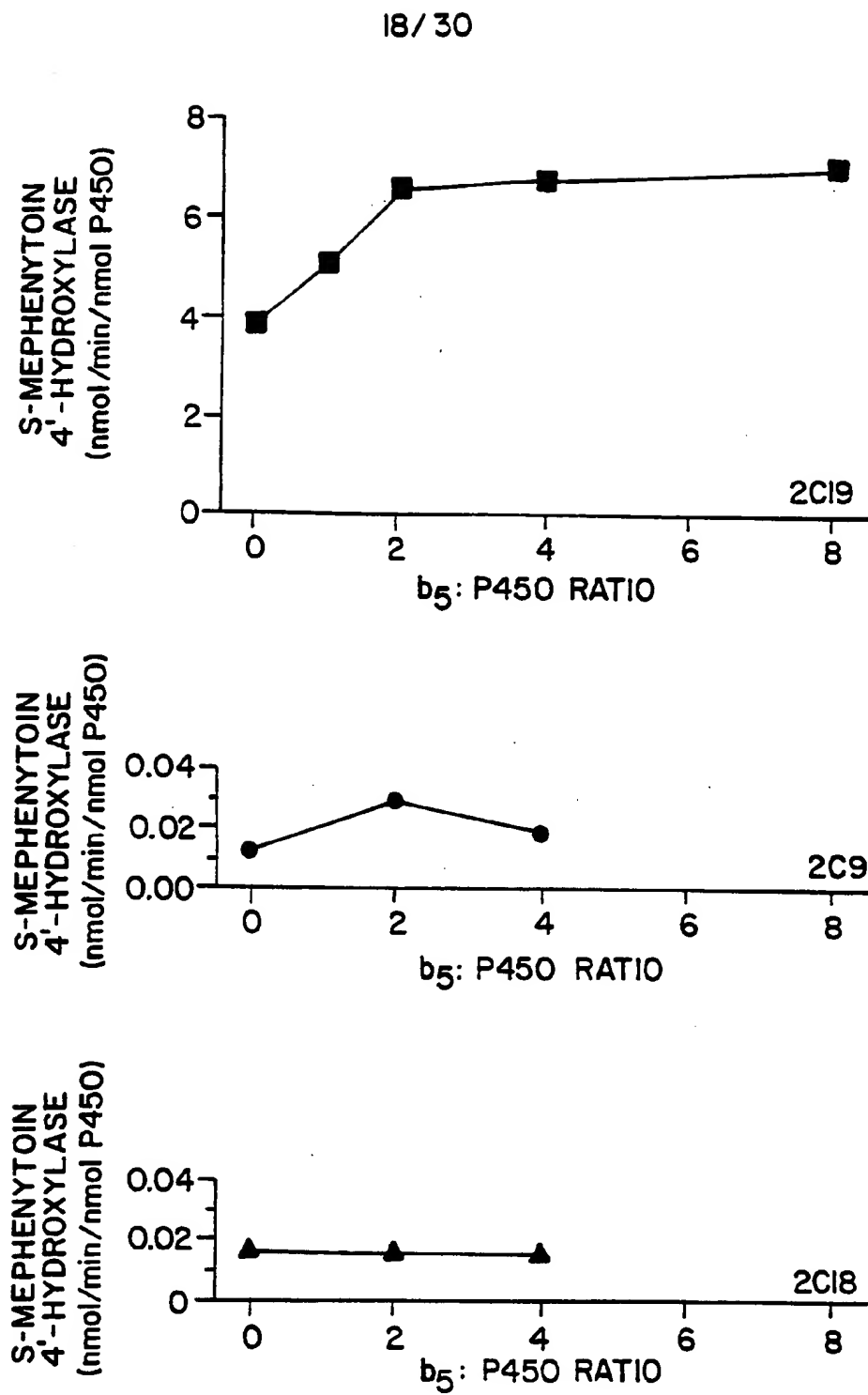
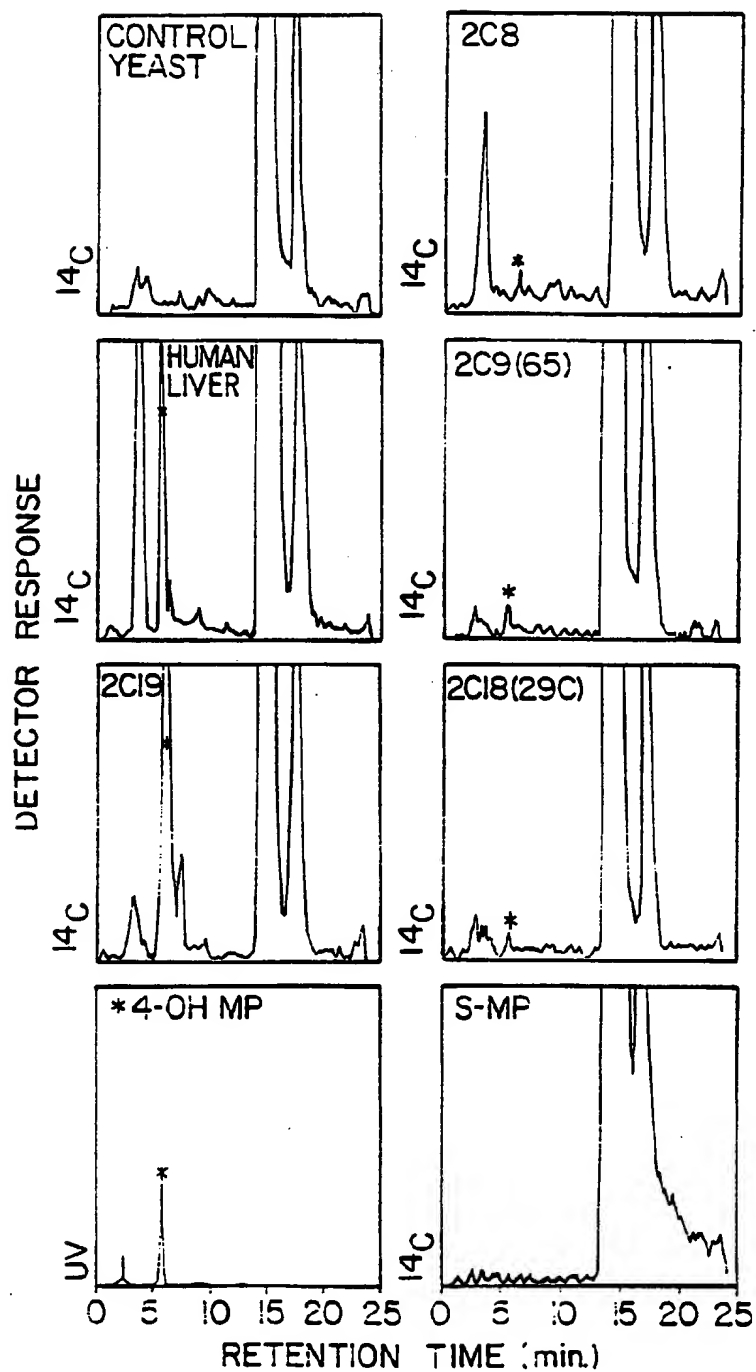


FIG. 8.

19/30

**FIG. 9.**

20/30

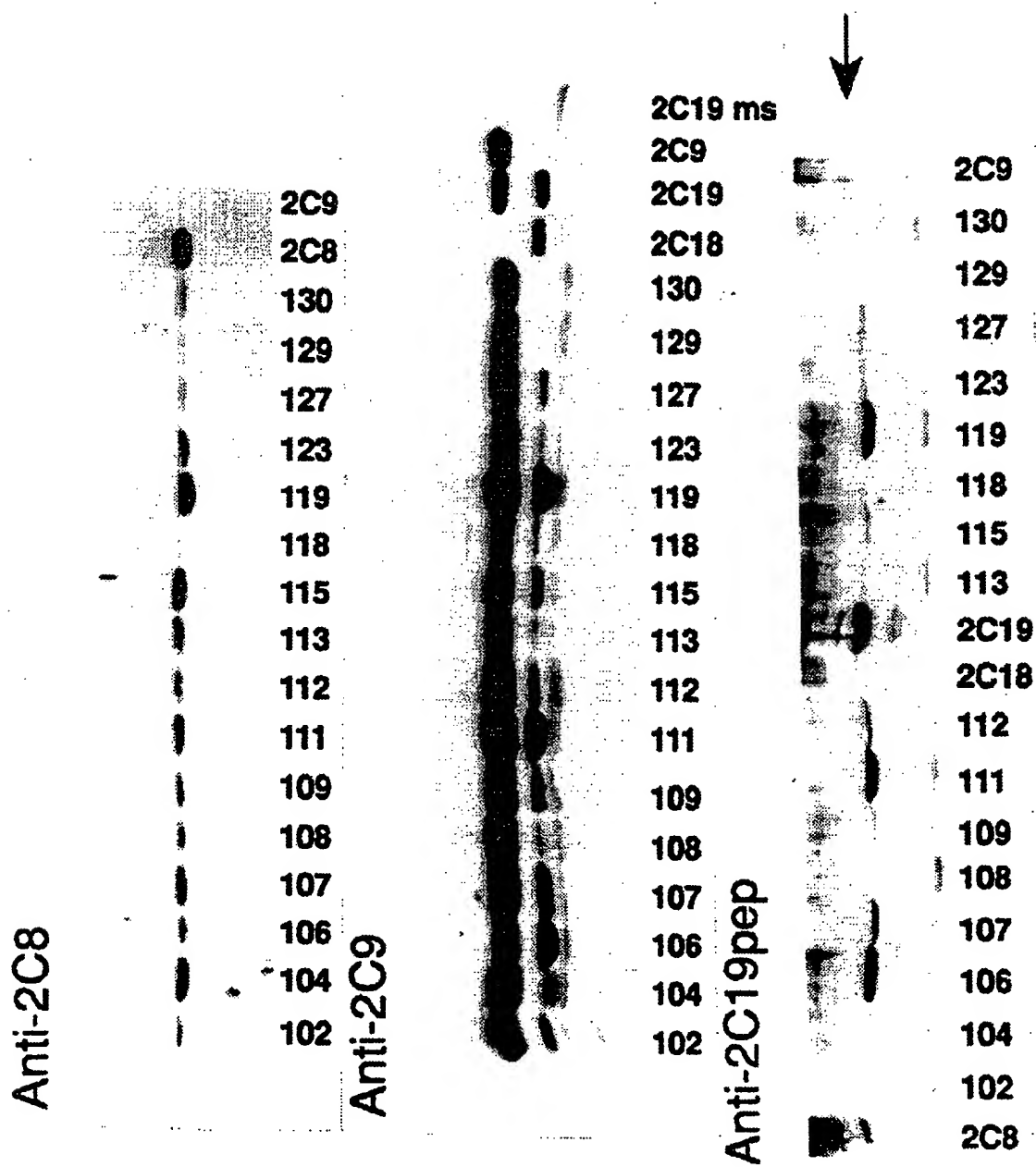


FIG. 10-1.

21 / 30

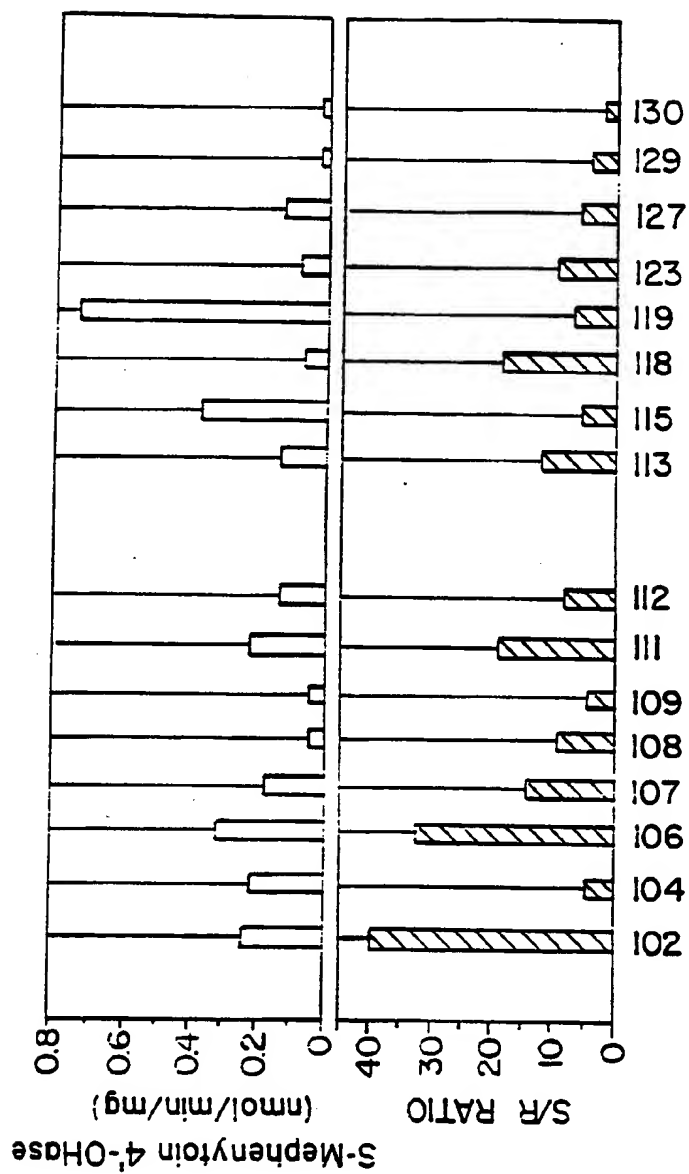
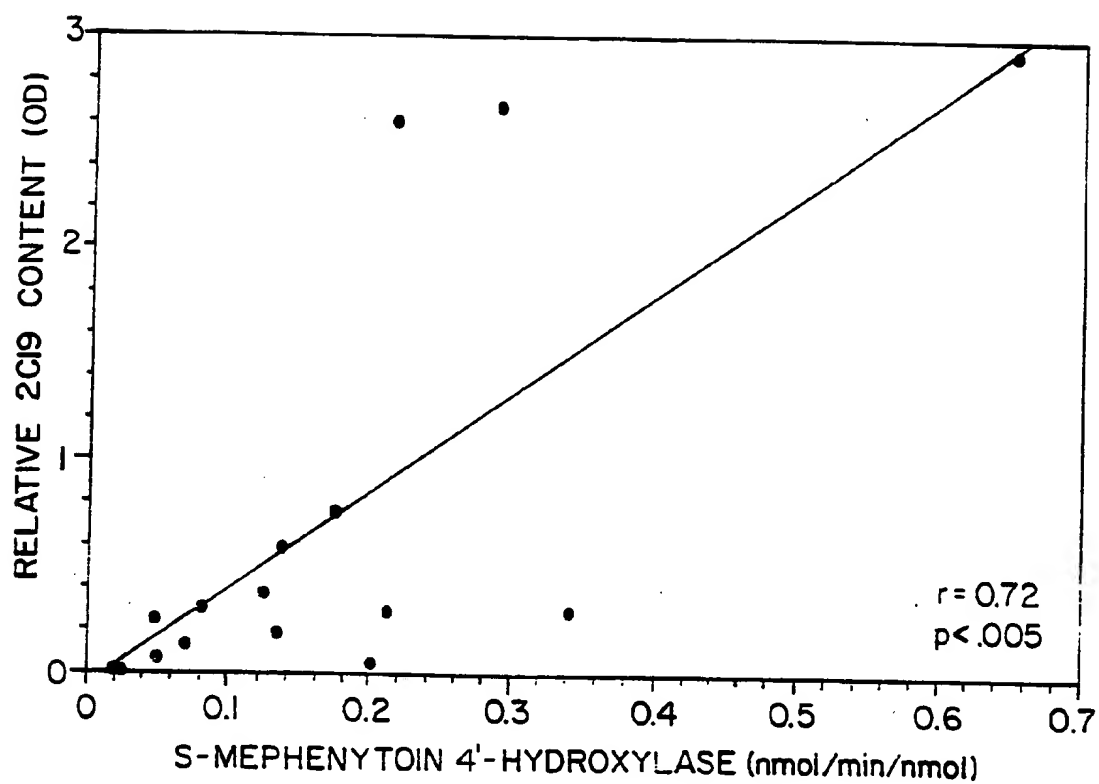


FIG. 10-2.

22/30

**FIG. II.**

23/30

Exon 4 | Exon 5

Leu Asn Glu Asn Ile Arg Ile Val Ser Thr Pro Trp Ile Gln Ile Cys Asn Asn Phe Pro Thr Ile Ile Asp Tyr
 600 ATTGAATGAAACATCAGGATTGTAAAGCACCCCTGGATCCAGATATGCAATAATTTTCCCACTATCATTTGATTAT
 |||||
 ATTGAATGAAACATCAGGATTGTAAAGCACCCCTGGATCCAG.....

Phe Pro Gly Thr His Asn Lys Leu Lys Asn Leu Ala Phe Met Glu Ser Asp Ile Leu Glu Lys Val Lys Glu
 676 TTTCCGGGGAACCCATAACAAATTACTTAAACCTTGCTTTATGGAAAGTGATATTTGGAGAAAAGTAAAGAA
 |||||
GAACCCATAACAAATTACTTAAACCTTGCTTTATGGAAAGTGATATTTGGAGAAAAGTAAAGAA
 Glu Pro Ile Thr Asn Tyr Leu Lys Thr Leu Leu Trp Lys Val Ile Phe Trp Arg Lys END *

Exon 5 | Exon 6

His Gln Glu Ser Met Asp Ile Asn Asn Pro Arg Asp Phe Ile Asp Cys Phe Leu Ile Lys Met Glu Lys Lys
 751 CACCAAGAAATCGATGGACATCAACAACCTCGGGACTTTATTGATGCTTCCTGATCAAAAATGGAGAAAGGAAAAG
 |||||
 CACCAAGAAATCGATGGACATCAACAACCTCGGGACTTTATTGATGCTTCCTGATCAAAAATGGAGAAAGGAAAAG

Gln Asn Gln Gln Ser Glu Phe Thr Ile Glu Asn Leu Val Ile Thr Ala Ala Asp Leu
 826 CAAAACCAACAGTCTGAATTCACATATTGAAAACCTGGTAATCAGCTGCAGCTGACTTAC NORMAL cDNA (2C19wt)
 |||||
 CAAAACCAACAGTCTGAATTCACATATTGAAAACCTGGTAATCAGCTGCAGCTGACTTAC ABERRANT cDNA (2C19m)

FIG. 12.

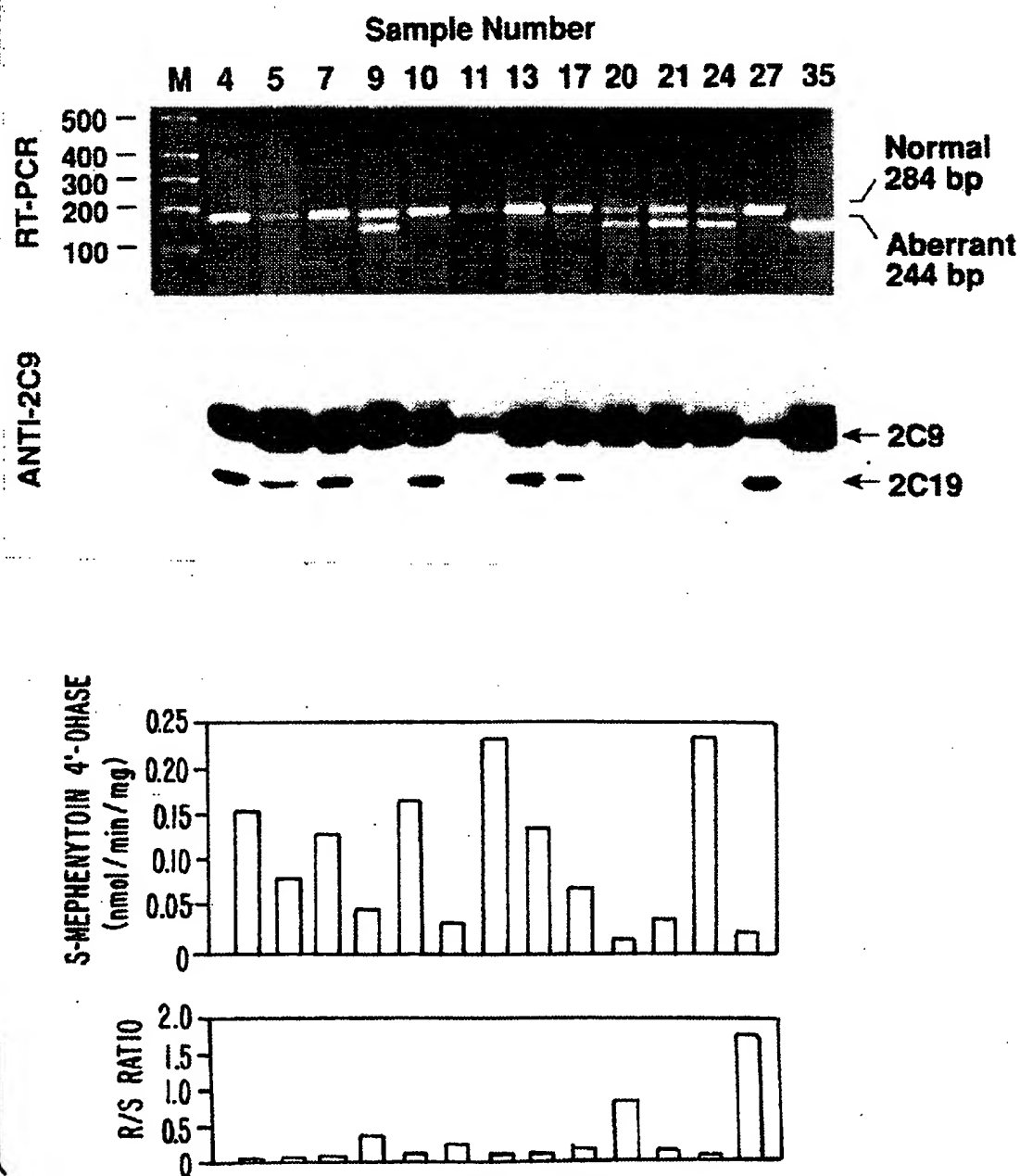
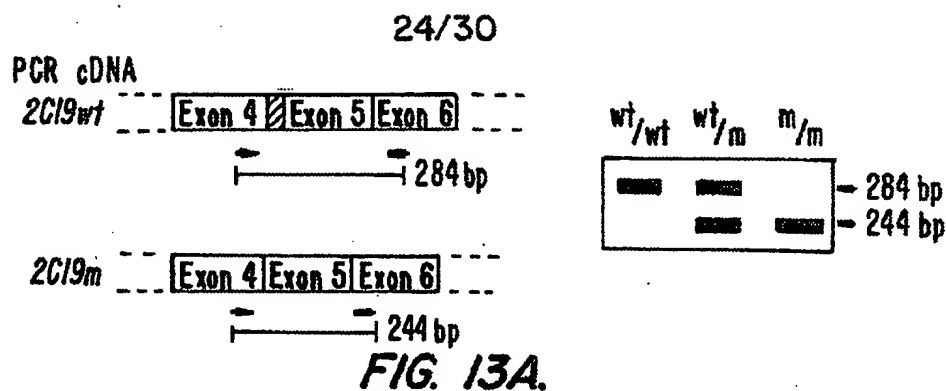
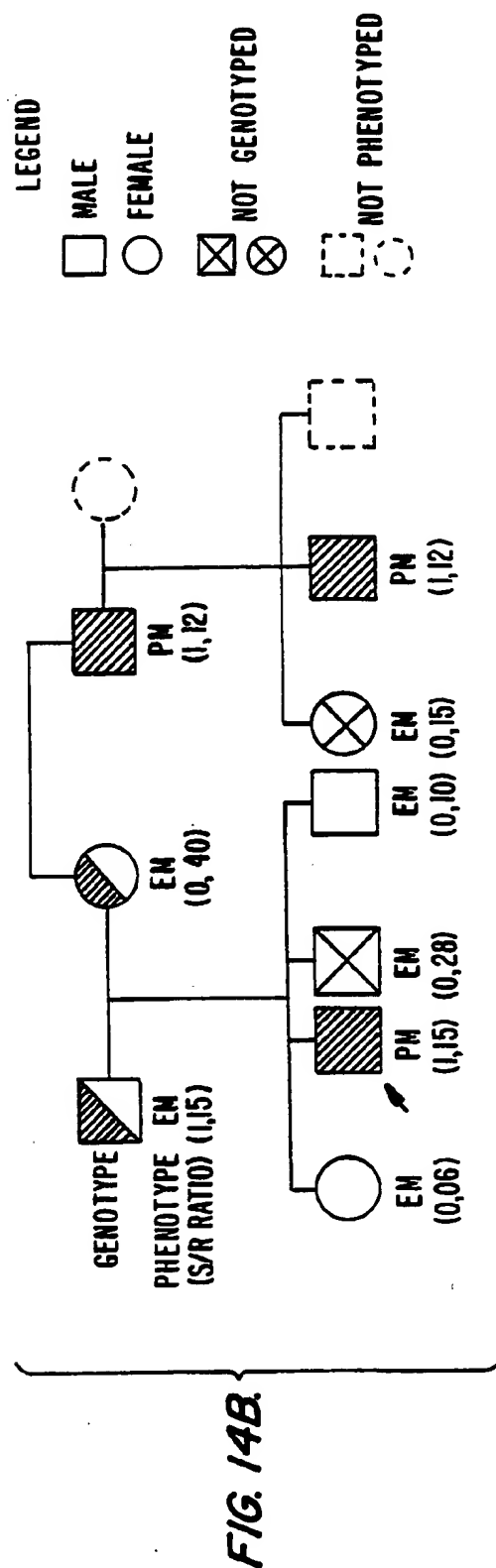
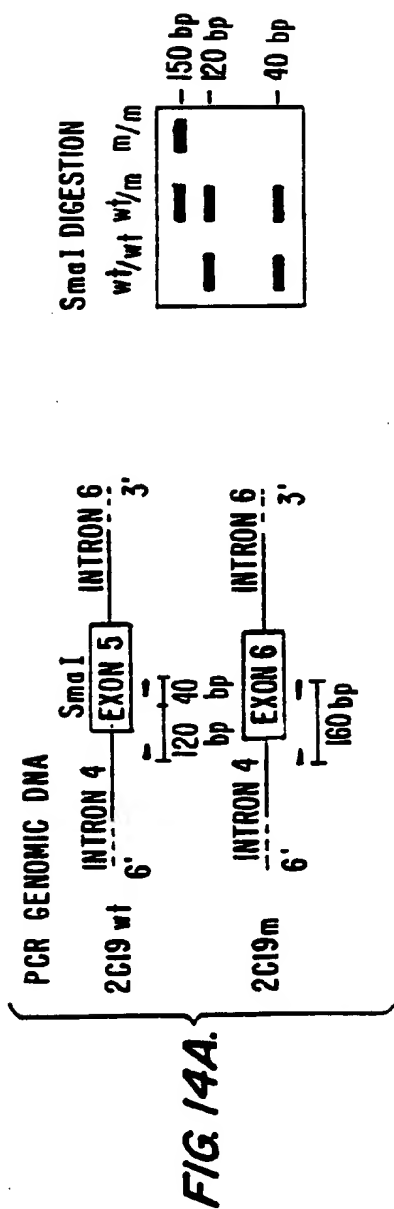
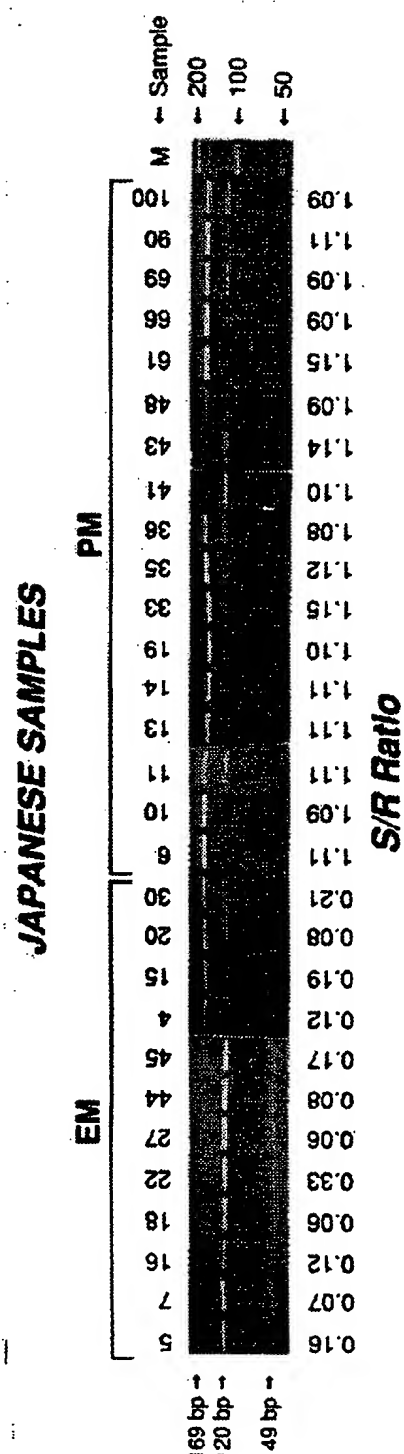
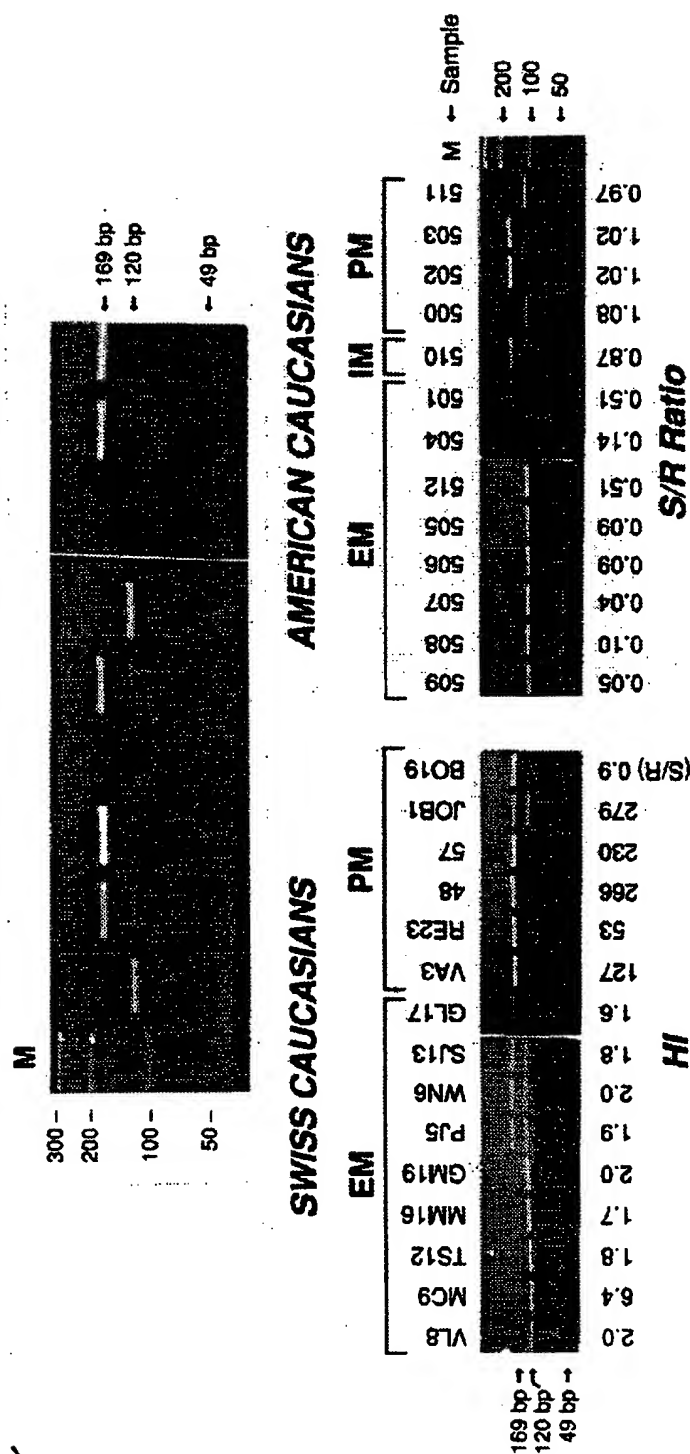


FIG. 13B. SUBSTITUTE SHEET (RULE 26)





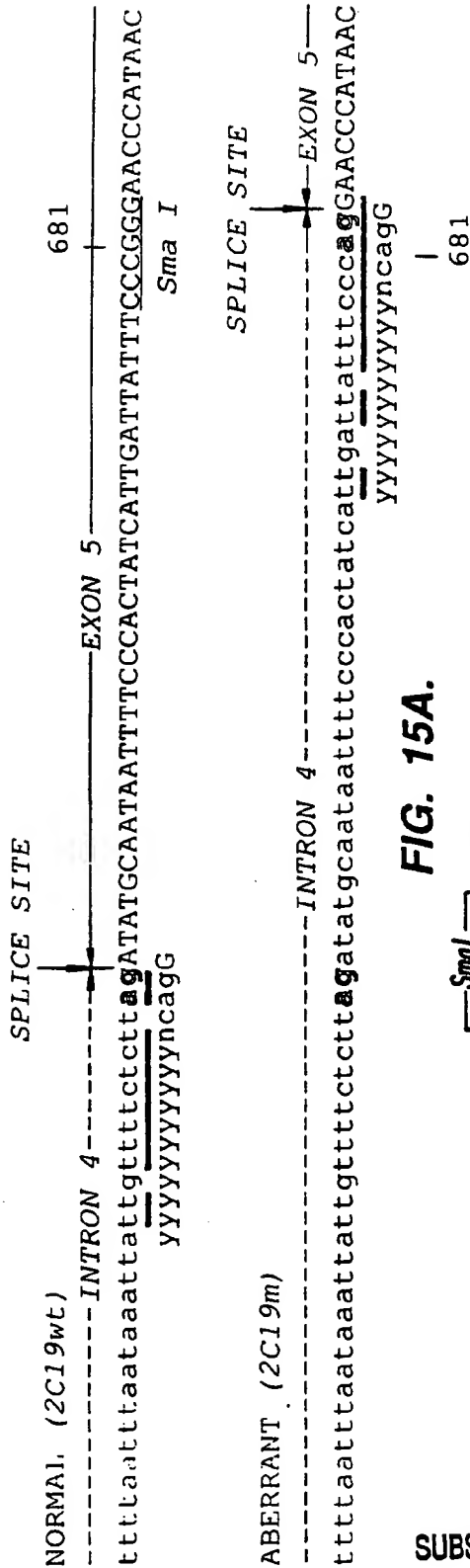


FIG. 15A.

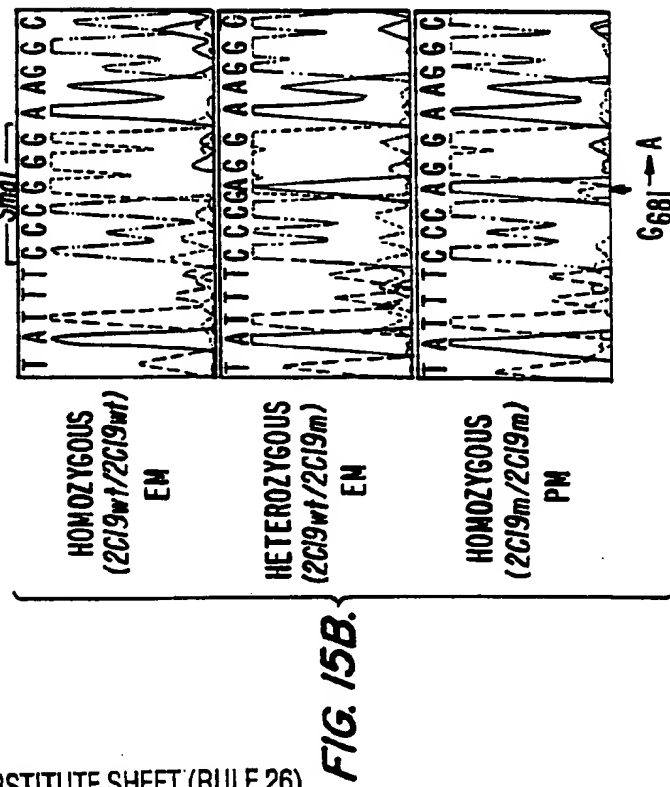


FIG. 15B.

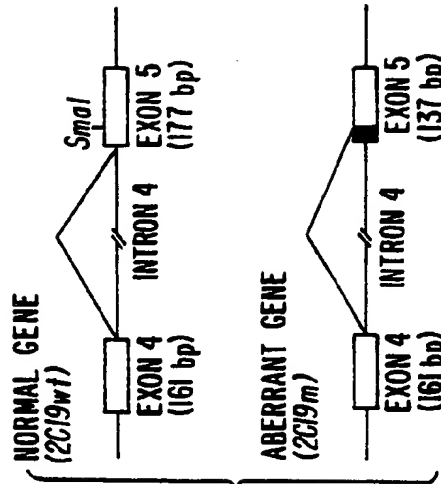


FIG. 15C.

28/30

ATGGTGATGT AGnAAntCAT nCCATCTTAT ATTTcNAGAG TGtAGAGGAG
 GATTGTTGnG GAAGTAAGAG GnnTAAGATA GAGATGCnTT TATACTATCC
 CAAGCAGGGA TrAGTCTAGG AAATGATTaT CGTCttTGAT TCTCTTGtCA
 GrAttTTCTT TCTCmnATCT TGtATAATCA GAGaatTACT ACACATGgAC
 AATrAarATT TCCCCnTCcA GAtAnACaAt ATATTTTATT TATATTTATA
 GTTTTAAATT ACAACCAGAG CTTGGCATAT TGTATCTATA CCTTTAATAA
 ATGCTTTTAA TTTAATAAAT TATTGTTTTc TCTTAGATAT GCAATAATTT
 TCCCACTATC ATTGATTATT TCCCGGGAAC CCATAACAAA TTAActTAAAA
 ACCTTGCTTT TATGGAAAGT GATATTTTGG AGAAAGTAAA AGAACACCAA
 GAATCGATGG ACATCAACAA CCCTCGGGAC TTTATTGATT GCTTCCTGAT
 CAAAATGGAG AAGGTAAAAT GTTAACAAAA GCTTAGTTAT GTGACTGCTT
 GCGTATkTGT GATTCATTGA CTAGTTGkGT GTTTACTACG GATGTTTAAC
 AGGTCAAGGA GTAATGCTTG AGAAGCATAT TTAAGTTTTt ATTGTaTGCA
 TGAATATCCA GTAAGCATCA TAGAAAATGT AAAATTAAnt TGtTAaATAa
 TTAGAaTACA TAGAAGAAAT tGTTtAGATA AATATnATCT ATCTGAACAA
 TAAGGATGTC AGGATAGGAA AAGCTCTGTT TCTGCAGCTT CCAGTGGAGA
 TCAGCACAGG AGGGAACCTA TTTTTT

FIG. 16.

29/30

agggaaaagacaaataggccggggatgnaaatttagcatgtgagcaacc wt
 ttanttaaccagctaggctgtaattgntaattcgagantaatgtnaaagt wt
 gatgtgttgattttatgcatgccnnaactcntttttgcttttaaggggagt wt
 cataggttaagatattacttaaaatttctaaactattattatctgttaact wt
aatatgaagtgttttatatctaatgtttactcatatttttaaattgtttc wt
 |||||
 atgaagtgttttatatctaatgtttactcatatttttaaattgtttc mutant

SerProCysAspProThrPheIleLeuGlyCysAlaP
 caatcatttagCTTCACCCTGTGATCCCACTTTCATCCTGGGCTGTGCTC wt
 |||||
 caatcatttagCTTCACCCTGTGATCCCACTTTCATCCTGGGCTGTGCTC mutant
 ^482

roCysAsnValIleCysSerIleIlePheGlnLysArgPheAspTyrLys
 CCTGCAATGTGATCTGCTCCATTATTTTCCAGAAACGTTTCGATTATAAA wt
 |||||
 CCTGCAATGTGATCTGCTCCATTATTTTCCAGAAACGTTTCGATTATAAA mutant
 [His]

AspFlnGlnPheLeuAsnLeuMetGluLysLeuAsnGluAsnIleArgIl
 GATCAGCAATTTCTTAACTTGATGGAAAAATTGAATGAAAACATCAGGAT wt
 |||||
 GATCAGCAATTTCTTAACTTGATGGAAAAATTGAATGAAAACATCAGGAT mutant

eValSerThrProTrpIleGln
 TGTAAGCACCCCCTGGATCCAGgtaaggacaagttttgtgcttcctgaga wt
 |||||
 TGTAAGCACCCCCTGAATCCAGgtaaggacaagttttgtgcttcctgaga mutant
 End ^642

aaccacttacagtctttttttctgggaaatccaaaattctatattgacca wt
 |||||
 aaccacttacagtctttttttctgggaaatccaaaattctatatt mutant

agccctgaagtacatttgtgaatactacagtcttgccctagacagccatgggggt wt
 gaatatctggaaaagatggcaaagntctttattttatgcacaggaaatgaata wt
 tcccaatatagatcaggcttctaagccattagctccctgatcagtgttt wt

FIG. 17.

30/30

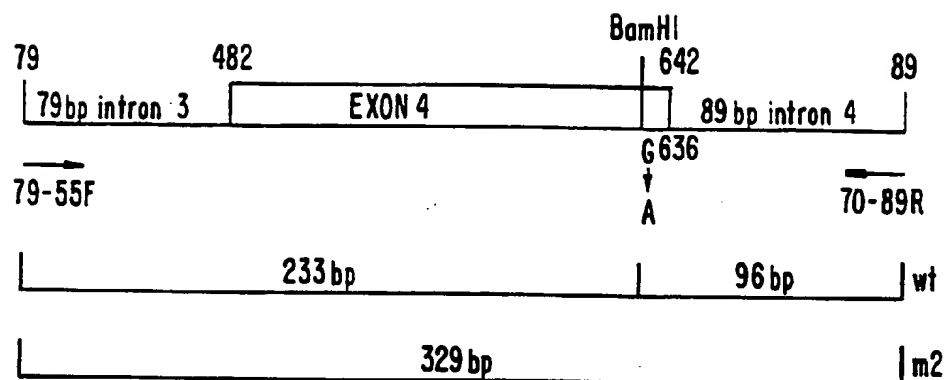


FIG. 18.

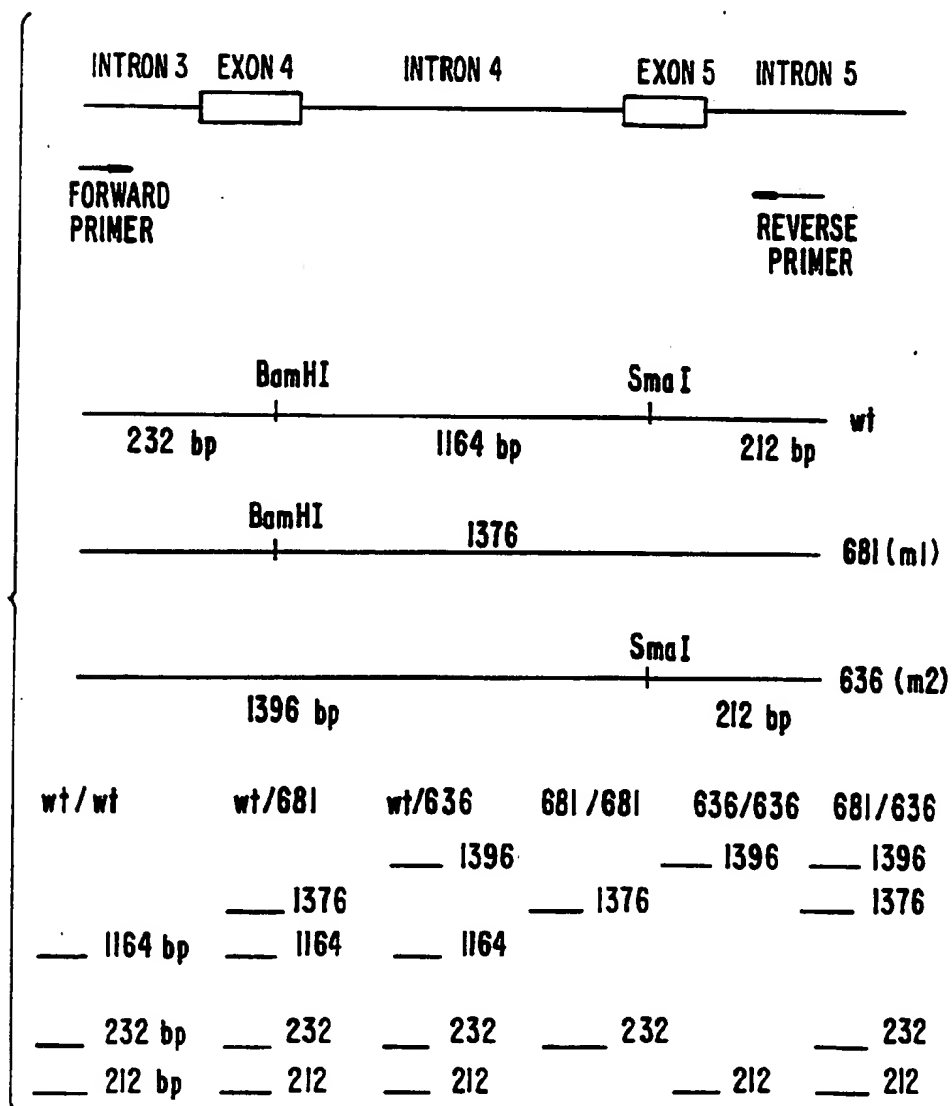


FIG. 19.

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05744

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/7.1; 435/6, 7.2, 69.1, 189, 192, 240.2, 252.3, 255, 320.1; 514/2; 536/22.1, 23.1, 23.2, 23.5, 24.3, 24.31, 24.33

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y ----- A	BIOCHEMISTRY, Vol. 30, No. 13 issued 02 April 1991, Romkes et al., "Cloning and Expression of Complementary DNAs for Multiple Members of the Human Cytochrome P450IIC Subfamily," pages 3247-3255, see entire document.	1-4 ----- 5-41
A	BIOCHEMISTRY, Vol. 26, No. 4, issued 1987, Wang et al., "Pseudomonas cepacia 3-Hydroxybenzoate 6-Hydroxylase: Induction, Purification, and Characterization," pages 1099-1104, see entire document.	5-41

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

24 JUNE 1995

Date of mailing of the international search report

09AUG 1995

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

Hyosuk Kim

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05744

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 261, No. 2, issued 15 January 1986, Shimada et al., "Human Liver Microsomal Cytochrome P-450 Mephenytoin 4-Hydroxylase, a Prototype of Genetic Polymorphism in Oxidative Drug Metabolism," pages 909-921, see entire document.	5-41
Y	J. BIOCHEM., Vol. 102, No. 5, issued 1987, Yasumori et al., "Nucleotide Sequence of a Human Liver Cytochrome P-450 Related to the Rat Male Specific Form," pages 1075-1082, see entire document.	4
Y	US,A, 4,943,521 (BLACKBURN ET AL) 24 July 1990, see entire document.	4
Y	US, A, 4,997,757 (SCHIESTI) 05 March 1991, see entire document.	4
Y	J. PHARMACOL. EXPER. THER., Vol. 241, issued 1987, Knodell et al., "Hepatic Metabolism of Tolbutamide: Characterization of the Form of Cytochrome P-450 Involved in Methyl Hydroxylation and Relationship to in Vivo Disposition," pages 1112-1119, see entire document.	4
Y	BIOCHEMISTRY, Vol. 26, No. 4, issued 1987, Umbenhauer et al., "Cloning and Sequence Determination of a Complementary DNA Related to Human Liver Microsomal Cytochrome P-450 S-Mephenytoin 4-Hydroxylase," pages 1094-1099, see entire document.	1-4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/05744

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

C12Q 1/00, 1/68; C12P 21/06; C12N 1/16, 1/20, 5/00, 9/02, 9/08, 15/00; C07H 17/00, 19/00, 21/00; A61K 38/00, 49/00

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/7.1; 435/6, 7.2, 69.1, 189, 192, 240.2, 252.3, 255, 320.1; 514/2; 536/22.1, 23.1, 23.2, 23.5, 24.3, 24.31, 24.33

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CAS ONLINE, BIOSIS

search terms: cytochrome p450, p450 2c19, mephenytoin, p450iic or p450 iic, cytochrome p450iic, cytochrome p450 iic, cytochrome 2c19, salmonella, tester strain, mutagen, carcinogen, auxotrophic histidine, mammalian cell line, yeast

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.